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Characterization of Resistance to the Cry1F Toxin from *Bacillus thuringiensis* in Resistant Fall Armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) from Puerto Rico

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CHARACTERIZATION OF RESISTANCE TO THE CRY1F TOXIN FROM *Bacillus thuringiensis* IN RESISTANT FALL ARMYWORM, *Spodoptera frugiperda* (J. E. SMITH) (LEPIDOPTERA: NOCTUIDAE) FROM PUERTO RICO

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Transgenic corn expressing Cry1F protein from *Bacillus thuringiensis* Berliner has been registered for *Spodoptera frugiperda* control since 2003. Unexpected damage to Cry1F corn was reported in 2006 in Puerto Rico and Cry1F resistance in *S. frugiperda* was documented. The inheritance of Cry1F resistance was characterized in a *S. frugiperda* resistant strain from Puerto Rico which displayed >387-fold resistance to Cry1F. Concentration-response bioassays of reciprocal crosses of resistant and susceptible parental populations indicated that resistance is recessive and autosomal. Bioassays of the backcross of the F_1 generation crossed with the resistant parental strain suggest that a single locus is responsible for resistance. Cross-resistance experiments indicated no significant Cry1F cross-resistance to Cry1Aa, Cry1Ba and Cry2Aa. Vip3Aa was effective against both strains indicating no cross-resistance with Cry1F. In contrast, significant cross-resistance (< 20-fold) was observed for Cry1Ab and Cry1Ac. An F_1 screen was performed to measure the frequency of Cry1F resistant alleles in 2010 and 2011. A total frequency of resistant alleles of 0.13 and 0.02 was found for Florida and

Texas populations respectively, indicating resistant alleles could be found in U.S. populations.

Fitness cost estimates associated with resistance suggest that heterozygous and homozygous resistant insects are equally fit with susceptible insects. This may affect initial allele frequencies in field populations and persistence in resistant populations (e.g. Puerto Rico).

Behavior experiments suggest that there is not a strong difference between resistant and susceptible phenotypes in *S. frugiperda* and *O. nubilalis.* However, behavioral differences were observed between species. *O. nubilalis* exhibited increased movement between leaf discs, with susceptible neonates avoiding Cry1F tissue. In contrast, *S. frugiperda* selected plant tissue regardless of the presence of Cry1F, suggesting that refuge in a bag might be a suitable strategy for this pest. Understanding resistance in field-derived resistant populations will provide information for better risk assessments, improve predictions of resistance and maximize the benefits of current and future generations of transgenic crops.

To my parents for encouraging me to follow my dreams

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction and Literature Review

Introduction

Spodoptera frugiperda (J. E. Smith, 1797) is an important pest of corn in the Tropics and throughout the U.S. as a late season pest in late-planted crops (Buntin 1986, Wiseman & Davis 1979, Mitchell *et al.* 1991). Larval feeding on corn indirectly affects grain production; in addition, damage on meristematic tissue can affect the architecture of the plant (Wiseman and Davis 1979, Buntin 1986). Late instar *S. frugiperda* can generate extensive feeding damage and when numerous defoliate and disperse in large numbers (Capinera 2000, Flanders *et al*. 2007). Although, *S. frugiperda* is an important pest of corn, it can affect other crops like sorghum, cotton, rice and different type of grasses (Buntin 1986, Capinera 2000).

Conventional chemical control strategies are inconsistent and often unsatisfactory to control *S. frugiperda* in field corn. Almost immediately after hatching, neonates move to the whorl of corn plants where they are protected from foliar insecticide sprays (Harrison 1986, Siebert *et al.* 2008b). In addition, regional populations of fall armyworm have developed resistance to several classes of insecticides including carbamates, organophosphates and pyrethroids (Adamczyk *et al*. 1999). Recently, corn, *Zea mays* L., expressing the Cry1F toxin from *Bacillus thuringiensis* Berliner has been developed for control of *S. frugiperda*. Corn hybrids containing Cry1F has been commercially available since 2003 and is marketed as Herculex® I *Insect Protection* (transformation event TC1507).

Even though TC1507 corn has been commercially available in the U.S. since 2003, in Puerto Rico the event has been grown since 1998 for experimental plots, hybrid development and parental seed production (Buntin 2008). Unexpected damage to Cry1F maize hybrids was reported in 2006 in Puerto Rico and high levels of Cry1F resistance in *S. frugiperda* was subsequently reported (Matten *et a*l. 2008, Tabashnik *et al*. 2009). Storer *et al.* (2010) confirmed the high-level of resistance to Cry1F, and described the resistance as autosomal and recessive. *S. frugiperda* resistance represents one of four species with documented field-evolved resistance to *Bt* crops. Field resistance occurred after four years of commercialization, making it the fastest documented case of field-evolved resistance to a *Bt* crop, and the first case of resistance leading to withdrawal of a *Bt* crop from the marketplace (Tabashnik *et al*. 2009). Multiple factors are thought to have contributed to *S. frugiperda* resistance evolution to Cry1F in Puerto Rico. Nevertheless, more studies are necessary to understand how resistance evolved in Puerto Rico, to determine the risk of field resistance in the U.S., and to establish better resistance management tactics for *S. frugiperda*.

S. frugiperda field resistance gives us an opportunity to more rigorously test the correspondence between evidence and theory. Better documentation and analysis of field-evolved resistance promotes the scientific basis for improving resistant management strategies. Incorporating an enhanced understanding of observed patterns of field evolved resistance into future resistance management strategies can help us minimize drawbacks and maximize the benefits of current and future generations of transgenic crops (Tabashnik *et al*. 2009).

Literature Review

Fall armyworm

Biology

Fall armyworm, *S. frugiperda* is an endemic insect of the western hemisphere, distributed from North America to Argentina (Sparks 1979, Capinera 2000). Fall armyworm is an important corn pest in the Tropics and throughout the United States as a late season pest in late-planted crops (Wiseman and Davis 1979, Buntin 1986, Mitchell *et al.* 1991). Because *S. frugiperda* does not diapause in winter, it is vulnerable to freezing temperatures, and in North America it normally overwinters in the subtropical climates of southern Florida and southern Texas-Mexico (Sparks 1979, Buntin 1986, Mitchell *et al*. 1991). Fall armyworm populations reinvade much of the continental United States and Canada annually during the summer months (Mitchell *et al*. 1991).

The fall armyworm life cycle is completed in about 30 days in the summer, about 60 days in spring and autumn, and 80 to 90 days during winter (Sparks 1979, Capinera 2000). The number of generations per year varies depending of the area. In tropical regions, fall armyworm has12 generations per year, but in the United States the number of generations can fluctuate between one generation, in New York and Minnesota, to 10 in the coastal areas of Florida (Capinera 2000). A female moth can lay up to 1500 eggs with as many as 100 to 200 eggs/mass (Sparks 1979, Capinera 2000). Egg masses are typically oviposited on the lower side of leaves, but if the population density is high, oviposition can occur on all plant parts (Sparks 1979). There are typically six instars. The final instars descend from the plant and pupate in the soil. Larvae construct an oval

cocoon by tying together particles of soil with silk (Sparks 1979, Capinera 2000). Adults are nocturnal with activity beginning in the afternoon for host plant searching. Females then begin calling males or laying eggs. Females release pheromones to attract males and copulate more than once (Sparks 1979). Adults are strong fliers and pest spread northward has been estimated at 300 miles/generation in some years (Sparks 1979).

S. frugiperda displays a wide host range with over 80 plants recorded from 23 different families (Pashley 1988, Capinera 2000). The most frequently consumed plants are corn (*Z. mays*), cotton (*Gossypium hisutum* L.), sorghum (*Sorghum vulgare* Pers.), rice (*Oryza sativa* L.), and bermudagrass (*Cynodon dactylon* Pers.) (Buntin 1986, Pashley 1988, Meagher and Gallo-Meagher 2003). Field crops frequently injured by fall armyworm include corn, alfalfa, barley, bermudagrass, buckwheat, cotton, clover, oat, millet, peanut, rice, ryegrass, sorghum, sugar beet, sudangrass, soybean, sugarcane, timothy grass, tobacco, and wheat (Capinera 2000).

Population genetics studies

There are two strains of *S. frugiperda* based on their host plant preference (corn and rice). The corn strain feeds primarily on corn but also on cotton and sorghum. The rice strain feeds predominantly on rice, bermudagrass, and johnsongrass (Pashley 1986, Nagoshi and Meagher 2003a, 2004, Prowell *et al.* 2004). Host strains have been identified in different countries including the United States (Pashley 1986, Lu *et al*. 1992, 1994, Meagher and Gallo-Meagher 2003, Nagoshi and Meagher 2003a, 2003b, 2004, Prowell *et al.* 2004, Nagoshi *et al.* 2007), Puerto Rico, Dominican Republic, Jamaica, Costa Rica, Mexico, French Guyana, Ecuador (Prowell *et al.* 2004), Brazil (Busato *et al*.

2004, Martinelli *et al*. 2006, Nagoshi *et al.* 2007), Argentina (Clark *et al.* 2007), and Colombia (Saldamando and Velez-Arango 2010). The strains are indistinguishable morphologically in larvae and adults (Pashley 1988) but differ in their genetic constitution at a number of molecular markers (Pashley 1986, Lu *et al*. 1992, Lu and Adang 1996, McMichael and Prowell 1999,Levy *et al*. 2002, 2003b, Clark *et al*. 2007, Nagoshi *et al*. 2008), and in their physiology (Prowell 1988, Quisenberry and Whitford 1988, Whitford *et al*. 1988, Veemstra *et al*. 1995, Prowell *et al*. 2004).

Recently, a novel method involving mitochondrial haplotype ratios of the corn strain has been used to study migration of fall armyworm in North America, Puerto Rico and Brazil (Nagoshi *et al.* 2010). This method uses DNA sequencing information from a portion of the mitochondrial *cytochrome oxidase I* (COI) gene used for DNA barcoding analysis. Fall armyworm corn strain populations can be subdivided in four COI haplotypes classes (CS-h1 - CS-h4) defined by single-base polymorphisms at two sites (Nagoshi *et al.* 2007). Proportions of two of the haplotypes (CS-h4/CS-h2) showed ratios of <0.5 for Texas and Brazil, and >1.5 for Florida. These different ratios suggest the existence of a reproductive barrier between Florida and Texas that prevent the homogenization of the haplotype ratios over time (Nagoshi *et al*. 2008). A different study found that corn strain fall armyworm from Puerto Rico and Florida displayed similar CS-h4/CS-h2 ratios. Indicating that Puerto Rico populations are genetically more similar to populations in Florida than those in Brazil or Texas (Nagoshi *et al.* 2010). Additionally, collections from corn producing areas in southern, central, and eastern United States were used to map the geographical distribution of fall armyworm haplotypes (Nagoshi *et al.* 2012). These haplotype profiles allowed the development of a detailed description of the

annual northward movement of fall armyworm. Results from this research suggest that Texas populations migrate northward into Kansas, Nebraska, Iowa, Minnesota, Illinois and eastward to Pennsylvania. In contrast, migration from Florida is limited to the Southern Atlantic coastal states and is restricted to regions of the east Appalachian Mountain range (Nagoshi *et al.* 2012). Overlap between populations originating from Florida and Texas appears to be limited to small areas north and south of the primary elevations of the Appalachians, suggesting limited opportunities for genetic exchange. Findings of this study were suggested to have implications for estimating the potential threat of invasive populations carrying deleterious alleles that might become established in Florida (Nagoshi *et al*. 2012)

Damage and management

S. frugiperda larvae typically cause damage by consuming foliage. Young larvae initially consume leaf tissue from one side, leaving the opposite epidermal layer intact. By the second or third instar, larvae begin to make holes in leaves, and eat from the edge of the leaves inward. In later instars, larval densities are usually reduced to one to two per plant due to the cannibalistic behavior (Pitre and Hogg 1983, Capinera 2000). Sixth instar larvae can eat more than all the other stages combined and thus cause almost all the plant damage. During the final 2-3 days of feeding, armyworms consume 80% of the total foliage consumed during their entire development (Sparks 1979, Capinera 2000, Knuston 2009). Extensive feeding damage can occur often removing all foliage and leaving only the leaf midribs and stalks of plants (Capinera 2000, Flanders *et al*. 2007). Larvae can

also burrow into a plant's growing point, destroying the growth potential of the plant (Capinera 2000).

S. frugiperda is an important pest of corn in Florida and Latin America. In other parts of the U.S.it is a sporadic but devastating pest with outbreaks at irregular intervals (Sparks 1979). The attack of larvae during the vegetative growth indirectly affects the grain production because of the reduction of the photosynthetic area. Additionally, larvae can damage the meristematic tissue modifying the architecture of the plant and can burrow into the ear, feeding on kernels in the same manner as corn earworm, *Helicoperva zea* (Boddie) (Wiseman and Davis 1979, Buntin 1986, Capinera 2000). Fall armyworm can also affect other crops like sorghum, cotton, rice and different type of grasses, although it is considered as a secondary pest of these crops (Buntin 1986, Capinera 2000).

Different strategies have been used to manage fall armyworm including cultural practices, enhancement of natural enemies, conventional *Bt* insecticides, and *Bt* crops (corn and cotton). Cultural practices employed include early planting in the southern states, use of early maturing varieties, early harvest, planting of tolerant varieties and crop rotation. Conventional insecticides used against fall armyworm are primarily pyrethroids, methomyl and carbaryl (Capinera 2000, Knuston 2009). However, conventional chemical control strategies are inconsistent and often unsatisfactory to control *S. frugiperda* in field corn due to movement into the whorl region of corn plant where they are protected from foliar insecticide sprays (Harrison 1986, Siebert *et al.* 2008b). In addition, regional populations of fall armyworm have developed resistance to

several classes of insecticides including carbamates, organophosphates, and pyrethroids (Adamczyk *et al.* 1999).

The most recent strategy to control fall armyworm has been the use of *Bt* transgenic corn and cotton (Siebert *et al*. 2008a, 2008b). Transgenic crops that produce toxins from *Bt* can control key pests, thereby reducing the dependence on chemical insecticide applications. Ingested *Bt* toxins kill susceptible insects by binding to and disrupting their midgut membranes. Corn expressing Cry1F, and cotton expressing Cry1Ab and Cry1F have been used for fall armyworm management (Siebert *et al*. 2008a, 2008b). Studies have demonstrated that corn and cotton hybrids containing Cry1F provide better fall armyworm control than corn hybrids producing Cry1Ab or cotton varieties containing Cry1Ac alone (Steward *et al.* 2001, Waquil *et al.* 2002, Buntin 2008, Siebert *et al.* 2008, Hardke *et al.* 2011). Dow AgroSciences (Indianapolis, IN) and Dupont Pioneer (Johnston, IA) developed corn hybrids that express Cry1F insecticidal protein of *B. thuringiensis* var. *aizawai* (Storer *et al*. 2010, 2012). Corn hybrids containing Cry1F have been commercially available since 2003 and marketed as Herculex® I *Insect Protection* (transformation event TC1507). It have been suggested that maize varieties producing Cry1F can be an important component of an overall management program for fall armyworm across a broad range of geographies (Siebert *et al*. 2008a).

Resistance Management to Crops Expressing *Bt* Toxins

Transgenic crops expressing toxins from *B. thuringiensis* used to control key insect pests have been widely deployed in the United States and globally since 1996

(Shelton *et al.* 2002, James 2009). However, since the inception of *Bt* transgenic crops, it has been recognized that extensive and prolonged exposure to *Bt* toxins could generate resistance in target pest populations reducing long-term utility of the technology (Gould 1988, 1994, 1998, Van Rie 1991, Mallet and Porter 1992, Roush 1994). Integrated resistance management (IRM) programs have been developed by government agencies in the United States and Canada to reduce the possibility of development of insect resistance and enhance strategies to manage resistance when it occurs (Gould 1998, EPA 2008). It has been suggested that a good resistance management strategy will sustain the efficacy of the toxin(s) for more than 10 years (EPA 1998).

The high-dose refuge strategy has been widely adopted to manage resistance evolution (Tabashnik *et al.* 2003). The refuge portion of the strategy requires that target pests have a refuge from toxins to maintain a source of susceptible alleles and decrease selection for resistance. The high-dose refuge strategy also involves the use of plants expressing a high-dose of *Bt* toxin that should kill more than 95% of heterozygotes carrying a resistant allele, thereby preventing heterozygotes from passing the resistant alleles to the next generation. The high-dose refuge strategy incorporates three basic assumptions; 1) resistance to *Bt* is recessive and controlled by a resistant allele at one locus; 2) the initial frequency of the resistant allele (R) in pest populations is low; and 3) random mating between resistant adults (RR) and susceptible moths (from refuges) keeps the R allele rare. Rare resistant survivors from *Bt* fields will more likely mate with abundant susceptible insects from refuge areas and heterozygotes will be killed by the high-dose expressed by plants (Georghiou and Taylor 1977, Gould 1998, Carrière and Tabashnik 2001, Tabashnik *et al*. 2003, 2009, EPA 1998, 2008).

An additional strategy to delay resistance is the use of second-generation *Bt* crops that produce two distinct *Bt* toxins that are active against the same pest. This approach, which is called a "pyramid", is expected to delay pest resistance most effectively when selection for resistance to one of the toxins does not cause cross-resistance to the other toxin (Zhao *et al*. 2005). Insect resistance theory predicts that resistance is likely to evolve more slowly in populations simultaneously targeted with multiple insecticides than populations exposed to single insecticides (Curtis 1985). Resistance against toxins with different modes of actions is rare in field populations (Gould 1998). The success of pyramided *Bt* crops includes the use of refuges and the following conditions for each toxin in the pyramid: recessive inheritance, low initial allele frequency, fitness costs associated with resistance, and incomplete resistance (Gould 1998, Zhao *et al*. 2005). The high dose strategy combined with the use of refuges of non-*Bt* crops and pyramids of different toxins is considered to be the best technical approach for managing resistance (Gould 1998, Bates *et al*. 2005).

Fitness of Resistance

The study of fitness costs associated with resistance to *Bt* toxins is important for understanding resistance evolution and for evaluating resistance management practices that prevent or mitigate resistance to transgenic crops in the field (Carrière and Tabashnik 2001). Newly arisen resistance traits are often assumed to be associated with a fitness cost. This assumption arises from the observation that resistance genes are rarely fixed in populations, and the maintenance of genetic polymorphisms is thought to be a result of counterbalanced selection pressures (Coustau *et al.* 2000). The general assumption is that

in the absence of an insecticide, resistant phenotypes are at a genetic disadvantage relative to susceptible phenotypes (McKenzie 1996); individuals carrying a resistant allele will have a reduced fitness relative to susceptible individuals on non-*Bt* hosts (Ferré and Van Rie 2002).

Resistance alleles are rare during initial stages of resistance evolution, appearing almost entirely as heterozygotes (Georghiou and Taylor 1977). Resistance traits are often assumed to be associated with fitness costs associated with resistance genes or with other loci closely linked to the resistance gene(s) (Gassmann *et al.* 2009). The relative fitness of heterozygotes influences response to selection and the rate of resistance evolution (Carrière and Tabashnik 2001). In most cases where resistance to *Bt* has been identified, there has been a relative rapid decline in resistance levels once selection pressure has been removed (Ferré and Van Rie 2002, Gassmann *et al.* 2009). Additionally, models show that fitness costs can help to delay resistance by selecting against *Bt*-resistant genotypes in refuges where insects are not exposed to *Bt* toxins (Tabashnik *et al*. 2003, Gassmann *et al.* 2009).

To evaluate how fitness costs will affect resistance management, the dominance of fitness cost must be analyzed using several fitness components of the insect life cycle that may reduce or enhance the effectiveness of the high dose refuge strategy (McKenzie 1996, Crespo *et al.* 2010). The pleiotropic effects of resistance alleles affect a variety of life history traits and can be detected as lower larval growth rate (Liu *et al*. 1999), survival (Groeters *et al.* 1994), or fecundity and mating success (Groeters *et al*. 1993, McKenzie 1996). Studies of fitness costs in the absence of selection can be conducted in the laboratory either by monitoring the stability of resistance (cage studies) or by

comparing one or more fitness components (e.g. survival, development rate, fecundity). Both types of studies are important because knowledge of global measurement of costs (cage studies) together with specific fitness traits affected by resistance could improve measures to delay resistance (Roush and McKenzie 1987, Roush and Tabashnik 1990, McKenzie 1996, Gassmann *et al*. 2009). Whether single generation or population cage studies are used, comparisons must be made between strains with common genetic background. For extrapolation to the field, genotypes used in the experiments should be field-derived if possible. If estimates of fitness costs are not made in a common genetic background differences ascribed to resistance genotypes may be due to strain origin and associated epistatic interactions that are independent of relative fitness values at the resistance locus (McKenzie 1996).

Fitness costs are generally considered from a conservative approach. The potential advantages to resistance management from fitness costs are limited as fitness costs have not always been associated with resistance alleles. In addition, the relationship between resistance genes and fitness costs may not always be straightforward (EPA 1998). Nearly all research on fitness costs of *Bt* resistance has been done in laboratories and greenhouses. Fitness costs could be magnified by stresses insects experience in the field but not in laboratories, and therefore fitness costs may be underestimated (Gassmann *et al*. 2009). Experiments that simulate field conditions are the next step for assessing fitness costs, particularly to evaluate how costs are influenced by ecological and environmental factors (Gassmann *et al*. 2009).

Larval movement

Because the high dose resistance management strategy is intended to reduce survival of heterozygous insects, if fields are planted to a mixture of *Bt* producing plants and non-*Bt* plants, there is a potential for heterozygous larvae to move off a *Bt* plant onto a non-*Bt* plant. If larva feeds on a *Bt* plant for a short period of time then moves to a non-*Bt* plant, it may receive a dose of toxin below that necessary to meet the definition of a high dose. This may allow survival of heterozygous larvae and potentially accelerate the development of resistance. Dominance strongly affects resistance evolution. When a resistance allele is rare, resistant homozygotes will be rarer. The increased survival of heterozygotes caused by movement would accelerate resistance development (Mallet and Porter 1992, EPA 1998). Computer simulations also indicate that lepidopteran larval movement from transgenic plants in seed mixtures could significantly influence the effectiveness of seed mixtures to delay resistance (Onstad and Gould 1998).

Behavioral studies of lepidopteran larvae have shown that field exposure to toxins present in *Bt* corn increases the likelihood of larvae moving between plants (Davis and Onstad 2000, Prasifka *et al*. 2009). This behavioral response has been reported in lepidopteran species on *Bt* plants including *Ostrinia nubilalis* (Hübner) (European corn borer) (Davis and Onstad 2000), *Plutella xyllostela* (L.) (diamondback moth) (Ramachandran *et al.* 1998), *Heliothis virescens* (F.) (Tobacco budworm) (Parker and Lutrell 1999), and *Tricoplusia ni* (Hübner) (cabbage looper) (Li *et al*. 2006). Similar results were found in *Spodoptera exigua* (Hübner) (beet armyworm) (Berdegué *et al*. 1996), *O. nubilalis* (Davis and Coleman 1997), and *Epiphyas postvittana* (Walker) (light brown apple moth) (Harris *et al.* 1997) exposed to Cry toxins in the laboratory.

Fewer studies have examined the behavioral responses to *Bt* toxins in resistant insects (Prasifka, *et al*. 2009, 2010). Studies on diet containing Cry toxins have shown that movement is similar between resistant and susceptible strains. Resistant and susceptible strains of *H. viriscens* (Gould and Anderson 1991), *E. postvittana* (Harris *et al.* 1997), *S. exigua* (Berdegué et al. 1996), and *O. nubilalis* (Huang *et al*. 2001) tended to avoid diet containing *Bt* toxins. Additionally, *E. postvittana* (Harris *et al.* 1997) and *S. exigua* (Berdegué *et al.* 1996) exhibited increased movement after exposure to *Bt* toxin. In contrast, experiments with Cry1Ab resistant *O. nubilalis* indicated that resistant and hybrid larvae did not avoid dietary Cry1Ab. Resistant larvae were more likely to be found on diet with *Bt* toxin, and showed reduced movement when exposed to Cry1Ab diet compared to susceptible larvae (Prasifka *et al*. 2009, 2010). *O. nubilalis* video-tracking observations predicted that resistant larvae are more likely to disperse onto adjacent non-*Bt* plants, but it seems to reflect greater survival after toxin exposure for resistant larvae rather than increased activity (Prasifka *et al.* 2010).

A variety of refuge placement options have been considered, including planting of the refuges in blocks, strips or seed mixtures (Bates *et al*. 2005). Blocks and in-field strips are the predominant refuge placements currently used for field corn in the U.S. because of studies indicating larval movement from plant to plant (EPA 2001, Ross and Ostlie 1990). Onstad *et al*. (2011) suggest that neither blocks nor mixtures are clearly superior. Block refuges are more likely to delay resistance, but present a risk because of adult pest behavior and lower compliance with IRM from farmers. Conversely, seed mixtures enhance IRM compliance and encourage random mating (Davis and Onstad 2000). However, seed mixtures make pest monitoring more difficult and may increase the

risk of resistance due to larval behavior and greater adoption of *Bt* corn (Davis and Onstad 2000, Onstad *et al*. 2011).

The use of mixtures for insect resistance management has been considered for at least two decades. Only recently has the U.S. Environmental Protection Agency approved a conditional registration of a seed mixture for corn rootworm IRM for a corn hybrid expressing only one toxin (EPA 2010). However, each species must be considered independently and IRM practices should not be expected to be suited for all species. (EPA 1998, Onstad *et al*. 2011). Consequently, specific information about the behavior of different pests is important to appropriately define the details of resistance management (Onstad *et al*. 2011). Particularly, understanding adult and larval movement between plants and the avoidance of toxins are necessary to better understand the feasibility of different refuge configurations (Ross and Ostlie 1990, EPA 1998,).

Resistance Monitoring

The ability to effectively detect the evolution resistance to a *Bt* crop before a control failure is an important component for resistance management strategies and a regulatory requirement for registering *Bt*-expressing hybrids in the United States (EPA 1998, 2002; ILSI 1998). To be useful, a resistance-monitoring program must be conducted within the context of the goal to maximize the effectiveness of the technology through time. Monitoring programs need to be efficient and should be linked to a realistic action plan that would be implemented in case of resistance development is identified (Siegfried *et al*. 2007). If resistance occurs in the fields, extensive monitoring will be needed to determine stability, cause, and geographical extent of resistance. Monitoring

techniques will also provide a means to test the effectiveness of resistance management programs (EPA 1998).

Monitoring focuses on key target pest species in which loss of sensitivity would significantly affect the utility of the technology. Different methods can be used for monitoring resistance. Regardless of the method used, accurate and reliable bioassay methods are an essential component of resistance detection and characterization (Siegfried *et al.* 2007). Each crop/insect combination requires a different sampling strategy as well as specific techniques for assessing *Bt* susceptibility (EPA 1998). In some cases, field-collected insects are pooled in large groups for mating in the laboratory to generate field-derived strains for bioassays. Alternatively, families derived from single wild gravid females or from single-pair crosses done in the laboratory can be tested separately using F_1 or F_2 screening procedures (Gould *et al.* 1997, Andow and Alstad 1998, Mahon *et al.* 2010).

Single pair mating increases the efficiency of detecting recessive resistance alleles to assess initial field frequency of resistant alleles and could also be used for monitoring changes in allele frequencies (Gould *et al*. 1997, Andow *et al.* 1998). These techniques have been used to estimate the upper-limit for *Bt*-resistant allele frequencies, which is important for assessing the utility of resistance management strategies (Siegfried *et al.* 2007). The F_2 and F_1 screen can also be used to verify assumptions of the high dose, refuge strategy for *Bt* crops which is critical for developing resistance management plans (Andow *et al.* 1998).

F_2 screenings are used to detect rare recessive resistant alleles (Andow and Alstad 1998). This methodology has the advantage of potentially detecting recessive resistance

alleles in the heterozygous state. It involves collecting large numbers of individuals from the field and establishing single-female family lines. The offspring of each collected female are inbred within family lines and the F_2 generation is screened at a discriminating dose. The inbreeding process allows potentially heterozygous offspring to mate with each other, generating an easily detectible fraction of homozygous resistant offspring (Andow and Alstad 1998).

In contrast, an F_1 screening involves crossing a field insect (of unknown genotype) to a homozygous resistant insect and screening the F_1 offspring for resistance with a discriminating dose. This test is only possible when a resistant laboratory strain is available (Gould *et al.* 1997, Liu *et al.* 2008, Yue *et al*. 2008, Mahon *et al.* 2010). If the field insect is heterozygous for a resistance allele, then 50% of the offspring from a cross with a resistant mate should be homozygous for the same allele and thus resistant to a dose of toxin that only homozygous individuals can tolerate. This method is far less labor intensive than F_2 screenings because it does not require insects to be reared to the F_2 generation in the laboratory. Thus, for less effort, more individuals can be scored using the F_1 screen (Gould *et al*. 1997).

Both the F_1 and F_2 screening methods are expected to provide the same estimates for the form of resistance present in the homozygous resistant tester colony. However, if alternative forms of resistance resulting from different genes are also present in the field population, they will be detected by F_2 tests but remain undetected by F_1 tests (Mahon *et al.* 2010). The main limitation of both F_1 and F_2 screenings is that they are labor intensive and the rearing requirements are expensive. In addition, limited by the number of sibling families that can be obtained from a single collection (Siegfried *et al*. 2007). Also, these

techniques are efficient for estimating the frequency of recessive single locus resistance alleles, but not for resistance determined by a combination of alleles at multiple loci (Stondola and Storer 2004).

Field-evolved resistance to *Bt* crops

Field-evolved resistance has been defined as a genetically based decrease in susceptibility of a population to a toxin resulting from exposure of the population to the toxin in the field (Tabashnik 1994, National Research Council 1996). Field-evolved resistance occurs when exposure of a field population to a toxin increases the frequency of alleles conferring resistance in subsequent generations. However, detecting alleles that confer resistance without demonstrating that their frequency increased does not constitute evidence of field-evolved resistance. The relationship between field-evolved resistance and field-control problems depends on many factors. Additionally, detection of field resistance does not automatically trigger large-scale removal of valuable varieties from the market place (Tabashnik *et al*. 2009).

More than a decade after the initial commercialization of *Bt* crops, most target pest populations remain susceptible. Field-evolved resistance has been documented in some a few instances including *S. frugiperda* to Cry1F corn in Puerto Rico (Matten *et al.* 2008, Storer *et al.* 2010), *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Cry1Ab corn in South Africa (Van Rensburg 2007), *H. zea* to Cry1Ac and Cry1Aa cotton in southeastern United States, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) to Cry1Ac cotton in India (Dhurua and Gujar 2011), and *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) to Cry3Bb1 corn in Iowa,

United States (Gassmann *et al.* 2011). In all cases, field resistance occurred when one or more requirements of the high-dose refuge strategy were not met (Huang *et al*. 2011). Incorporating the understanding of observed patterns of field resistance into future resistance management strategies will help to minimize drawbacks and maximize the benefits of current and future generations of transgenic crops (Tabashnik *et al*. 2009).

S. frugiperda **resistance in Puerto Rico**

TC1507 maize expressing Cry1F from *B. thuringiensis* var *aizawai* was registered in the United States in 2001 to control important lepidopteran pests, including *O. nubilalis*, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae) (southwestern corn borer), *H. zea* (Lepidoptera: Noctuidae) (corn earworm), and *S. frugiperda* (Siebert *et al*. 2008a, 2008b). TC1507 has been commercially available in the United States since 2003, but in Puerto Rico it has been grown since 1998 for experimental plots, hybrid development and parental seed production (Buntin 2008). Unexpected damage to Cry1F maize hybrids was reported in 2006 in Puerto Rico and high levels of Cry1F resistance in fall armyworm was subsequently reported (Matten *et a*l. 2008, Storer *et al*. 2010). Field-evolved resistance of *S. frugiperda* to *Bt* corn producing Cry1F occurred after 4 years of commercialization, making this the fastest documented case of field-evolved resistance to a *Bt* crop and the first case of resistance leading to withdrawal of a *Bt* crop from the marketplace (Tabashnik *et al*. 2009).

Storer *et al.* (2010) confirmed that field-control failures of TC1507 maize in Puerto Rico were associated with high-level resistance based on diet bioassays. The highest Cry1F concentration tested against the resistant population did not cause

significant mortality (10,000 ng Cry1F/cm^2), suggesting a resistance ratio in excess of 1000-fold. To evaluate inheritance, the F_1 progeny from reciprocal crosses of the susceptible and resistant populations were bioassayed, and the dose-response statistics were compared. Mortality and growth inhibition data from the susceptible, resistant and F_1 progeny were used to calculate dominance of resistance. The resistance to Cry1F was shown to be autosomal and highly recessive. Sensitivity of the resistant and susceptible colonies to Cry1Ab and Cry1Ac was also evaluated, and no indication of strong cross-resistance to either toxin was found (Storer *et al.* 2010, Storer *et al.* 2012).

Multiple factors are thought to have contributed to the evolution of resistance to Cry1F in *S. frugiperda* in Puerto Rico (Storer *et al.* 2011, 2012). Puerto Rico is an island that provides an isolated ecosystem subdivided by mountainous terrain. This restricts migration and dispersal restricted and enables local populations to respond to selection. In addition, the tropical environment of Puerto Rico allows year-round cultivation with multiple generations exposed to selection pressure in a single growing year. The long history of using formulated *Bt* insecticides for managing *S. frugiperda* in vegetables and seed corn, along with use of other *Bt* maize events that produce Cry1Ab may have also contributed selection. The affected lines were silage hybrids, not adapted to tropical conditions and lacked native resistance traits (Storer *et al.* 2012). The selection pressure in 2006 was likely to have been the most intense seen to date (Storer *et al.* 2011, 2012). Although is highly polyphagous with many crop and non-crop hosts in Puerto Rico, in 2006 severe drought conditions forced fall armyworm populations to become concentrated in irrigated crops, of which Cry1F maize was an important component. After resistance was reported in 2006, Storer *et al.* 2012 continued monitoring

populations in Puerto Rico and in southern areas of the mainland United States. The majority of collections from Puerto Rico continued to show high levels of Cry1F resistance whereas populations collected from the southern United States have remained susceptible to Cry1F and TC1507 maize.

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Research Objectives

Overall objective

To characterize field evolved resistance to Cry1F identified in a Puerto Rican population of *S. frugiperda* to validate and improve resistance management practices involving transgenic *Bt* corn.

Specific objectives

1. Quantify the level of resistance using bioassays with Cry1F toxin in artificial diet; determine the genetic basis of resistance (i.e., dominance, sex-linkage, and number of resistance genes), and estimate survival rates and dominance relationships.
2. Determine cross-resistance to other Lepidopteran-active *Bt* toxins with.
3. Determine frequency of resistance alleles in U.S. populations where the resistance has not been previously reported (Florida and Texas).
4. Identify the effects of the resistance on reproductive and physiological fitness.
5. Identify the effects of resistance on larval movement.

CHAPTER 2: Inheritance of Cry1F resistance, cross-resistance and frequency of resistant alleles in *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

Introduction

Transgenic crops expressing toxins from *Bacillus thuringiensis* Berliner (*Bt*) have been used widely since 1996 to control key insect pests (Shelton *et al.* 2002, James 2009). However, concern has been expressed that extensive and prolonged exposure to *Bt* toxins may select for resistance in target pest populations reducing the long-term utility of the technology. Understanding how resistance evolves is critical to developing effective resistance management programs that are necessary to sustain the technology (Gould 1988, 1994, 1998, VanRie 1991, Roush 1994, United States Environmental Protection Agency 2001, Tabashnik *et al*. 2003).

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is endemic to the Western Hemisphere and distributed from North America to Argentina (Sparks 1979). It is an important pest of maize and cotton throughout the neotropics and a late season pest throughout the U.S. (Buntin 1986, Wiseman and Davis 1979, Mitchell *et al.* 1991). *S. frugiperda* does not diapause and is vulnerable to freezing temperatures. Seasonal migrations to temperate regions of North America occur from overwintering populations in southern Florida, southern Texas and Mexico (Sparks 1979, Buntin 1986, Mitchell *et al.* 1991). Population genetics studies suggest limited genetic exchange between Florida and Texas fall armyworm populations (Nagoshi *et al*. 2010, Nagoshi *et al*. 2012). Texas populations migrate northward into Kansas, Nebraska, Iowa, Minnesota, Illinois and eastward to Pennsylvania. In contrast, migration from Florida appears to be limited to the

southern Atlantic coastal states and is restricted to regions east of the Appalachian Mountain range. Overlap between Florida and Texas populations appears to occur in limited areas north and south of the primary elevations of the Appalachians (Nagoshi *et al*. 2012). *S. frugiperda* exhibits two strains (corn and rice) based on host plant preference. The corn strain feeds primarily on corn but also on cotton and sorghum. The rice strain feeds predominantly on rice, bermudagrass, and johnsongrass (Pashley 1986, Nagoshi and Meagher 2004). The strains are indistinguishable morphologically in larvae and adults (Pashley 1988) but differ in their genetic constitution based on a number of different molecular markers (Pashley 1986, Levy *et al*. 2002, Nagoshi *et al*. 2007a), and in their physiology (Pashley 1988, Quisenberry and Whitford 1988, Whitford *et al*. 1988).

One of the more recent developments for managing fall armyworm populations has been the use of *Bt* transgenic corn, *Zea mays* L., expressing the Cry1F toxin (Siebert *et al*. 2008a, 2008b) which provides better control than hybrids producing Cry1Ab (Buntin *et al.* 2000, Steward *et al.* 2001, Waquil *et al.* 2002, Buntin 2008, Hardke *et al.* 2011). Corn hybrids that express the Cry1F insecticidal protein from *B. thuringiensis* var. *aizawai* were developed by Dow AgroSciences (Indianapolis, IN) and Dupont Pioneer (Johnston, IA). These hybrids have been commercially available since 2003 and marketed as Herculex® I *Insect Protection* (transformation event TC1507). This product has demonstrated satisfactory control of *S. frugiperda* and other important lepidopteran pests (Siebert *et al*. 2008a, 2008b). Although TC1507 maize hybrids were commercialized in 2003, in Puerto Rico Cry1F expressing maize was first grown in 1998 for hybrid development and parental seed production as well as efficacy trials (Buntin

2008). Unexpected damage to Cry1F maize hybrids was reported in 2006 in Puerto Rico and high levels of Cry1F resistance in fall armyworm was subsequently documented (Matten *et a*l. 2008, Tabashnik *et al*. 2009, Storer *et al.* 2010).

Cry1F resistance evolution among *S. frugiperda* populations from Puerto Rico represents one of the few instances of documented field-evolved resistance to transgenic *Bt* crops. Other species with reported field-developed resistance include *Busseola fusca* (Fuller) resistant to Cry1Ab maize in South Africa (Van Rensburg 2007), *Helicoverpa zea* (Boddie) resistant to Cry1Ac and Cry2Aa cotton in southeastern United States (Tabashnik *et al.* 2009, Huang *et al.* 2011), *Pectinophora gossypiella* (Saunders) resistant to Cry1Ac cotton in India (Dhurua and Gujar 2011), and *Diabrotica virgifera vigifera* LeConte resistant to Cry3Bb1 maize in Iowa United States (Gassmann *et al.* 2011). Field-evolved resistance of *S. frugiperda* to Cry1F maize occurred after only four years of commercialization, making this the fastest documented case of field-evolved resistance to a *Bt* crop and the first case of resistance leading to withdrawal of a *Bt* crop from the marketplace (Tabashnik *et al*. 2009).

Storer *et al.* (2010) confirmed that field-control failures of TC1507 maize in Puerto Rico were associated with a high-level of resistance. The highest Cry1F concentration tested against the resistant population (10,000 ng Cry1F/cm^2) did not cause significant mortality, suggesting a resistance ratio in excess of 1000-fold. To evaluate inheritance, the F_1 progeny from reciprocal crosses of the susceptible and resistant populations were bioassayed, and the dose-response statistics were compared. Mortality and growth inhibition data from the susceptible, resistant and F_1 progeny were used to calculate dominance of resistance. The resistance to Cry1F was shown to be autosomal

and highly recessive. Sensitivity of the resistant and susceptible colonies to Cry1Ab and Cry1Ac was also evaluated, and there was no indication of strong cross-resistance to these toxins (Storer *et al.* 2010, 2012).

Multiple factors are thought to have contributed to the evolution of resistance to Cry1F in *S. frugiperda* in Puerto Rico (Storer *et al.* 2011, 2012). Puerto Rico is an isolated island ecosystem that is subdivided by mountainous terrain. This terrain may restrict migration and dispersal and enable intense selection within local populations. In addition, the tropical environment of Puerto Rico allows year round cultivation of maize with multiple generations exposed to selection pressure in a calendar year. The long history of using formulated *Bt* insecticides for managing *S. frugiperda* in vegetables and seed corn, along with use of other *Bt* maize events that produce Cry1Ab may have also contributed selection. The affected lines were silage hybrids, not adapted to tropical conditions and lacked native resistance traits (Storer *et al.* 2012). Finally, although fall armyworm is highly polyphagous with many crop and non-crop hosts in Puerto Rico, in 2006 severe drought conditions forced fall armyworm populations to become concentrated in irrigated crops, of which Cry1F maize was an important component. The selection pressure in 2006 was likely to have been the most intense seen to date (Storer *et al.* 2011, 2012). After resistance was reported in 2006, Storer *et al.* 2012 continued monitoring populations in Puerto Rico and in southern areas of the mainland U.S. The majority of collections from Puerto Rico continued to show high levels of Cry1F resistance whereas populations collected from the southern U.S. have remained susceptible to Cry1F and TC1507 maize.

Although resistance to Cry1F has previously been characterized in a population from Puerto Rico (Storer *et al*. 2010), certain aspects of the resistance have yet to be addressed. In the present study, inheritance patterns of Cry1F resistance (dominance and number of loci) and cross-resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry2Aa and Vip3Aa were determined. A complete characterization of the inheritance of resistance allowed performing F_1 screenings to detect the frequency of resistant alleles in field populations outside of Puerto Rico (Florida and Texas). The results of this research have direct implications for *S. frugiperda* resistance management for Cry1F maize.

Materials and Methods

Insect Strains and Rearing

The Cry1F-selected strain was generated by Dupont Pioneer (Johnston, IA) and originated from several hundred field collected fall armyworm egg masses from Puerto Rico cornfields during October 2008 and January 2009. Egg masses were brought into the laboratory in Johnston, Iowa, where 826 neonates were selected by exposing them to TC1507 leaf discs. Only larvae that survived a four-day exposure (785 larvae) were maintained and used to establish the Cry1F-selected strain. The susceptible strain was purchased from BioServ (Frenchtown, NJ), and has been in continuous culture since November 1997 with regularly screenings to monitor for any changes in insecticide susceptibility. The BioServ strain, Cry1F-selected strain and field-collected larvae from Puerto Rico were identified as corn-strain. Strain identification was performed using a PCR amplification of a region of the mitochondrial COI gene with posterior digestion with EcoRV as described by Nagoshi *et al*. (2007b).

Both strains were maintained using rearing techniques adapted from Perkins (1979) with at least 200 adults randomly mating at each generation. Adults were placed in 31x 23 cm wired hermit crab cages (Florida Marine Research, Sarasota, FL) with diet placed in a cotton pad inside of the bottom of a 100 x 15 mm petri dish (Fisherbrand, Waltham, MA) and replenished daily. Adult diet consisted of stale beer, ascorbic acid, propionic acid and aureomycin (Perkins 1979). Adults were allowed to mate and laid eggs on wax paper. Eggs were harvested daily and placed in 100 x 15 mm petri dishes with moistened filter paper until hatching. Larvae were reared on multispecies lepidopteran diet (BioServ, Frenchtown, NJ). Neonates were placed on shredded diet and allowed to growth until third instar. Approximately 300 third instar larvae were individually transferred to 1 oz. translucent polystyrene soufflé portion cups (Solo Cup Company, Lake Forest, IL) with 4.5 ml of diet to minimize cannibalism. Pupation and adult emergence occurred within the cups. Emergent adults were transferred daily to mating cages.

Bt **Toxins**

The Cry1F used in diet bioassays was expressed in BtG8 cells grown in CYS2 media with tetracycline and grown for 6 days at 30°C. Cells were harvested by centrifugation and the pellets were washed 5 times with 0.5 M NaCl and twice with water. Washed pellets were stored at -20°C. Pellets were lysed with 50mM sodium carbonate pH10, 10mM DTT overnight at 4°C. The lysate was concentrated with Millipore (Billerica, MA) concentrator devices (100,000 molecular weight cut off, MWCO) to ~12.5 mg/ml and dialyzed against 50mM Na Carbonate/Na Bicarbonate

pH10 using 25K MWCO dialysis tubing. Aliquots of 5mg and 20mg were made, which were flash frozen in LN2, and then lyophilized.

Cry toxins used for cross-resistance experiments were prepared from fermentation of recombinant *Escherichia coli* (Migula) strains transformed to express Cry1Aa (ECE52), Cry1Ab (ECE53), Cry1Ac (ECE54), Cry1Ba (ECE128) and Cry2Aa (ECE126). The strains were obtained from the *Bacillu*s Genetic Stock Center of The Ohio State University (Columbus, OH). Recombinant *E. coli* cultures were grown at 37°C for 48 h in Luria-Bertani media. Protoxins were obtained from *E. coli* fermentation products following the method described by Lee *et al.* (1992). Toxin preparations were quantified by desitometric quantification (Crespo *et al*. 2008) of the 60-65 kDa peptides after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared to a standard curve for bovine serum albumin (BSA). Endotoxins were stored at -80°C (Tan *et al.* 2011).

Vip3A used was from a single source of lyophilized Vip3Aa19 protein supplied by Syngenta Biotechnology (Research Triangle Park, NC). Vip3A protein was produced through an *E. coli* expression system and the protein was purified by anion exchange chromatography prior to lyophilization. Purity was assessed/quantified by Sypro Orange-stained SDS-PAGE. Lyophilized protein was kept frozen until use.

Bioassays

Bioassays were performed based on the methods described by Marçon *et al*. (1999) in 128-well bioassay trays (CD International, Pitman, NJ). One ml of European corn borer wheat germ-based diet (Lewis and Lynch, 1969) was dispensed into each well

and allowed to solidify. Seven concentrations of the toxin were used for LC_{50} determinations. Dilutions were made in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading on the diet surface. Each well was surface treated by applying 30 µl of the appropriate concentration. The negative control consisted of wells treated with 30 µl of 0.1% Triton-X 100.

The treated wells were allowed to air dry, and one randomly selected *S. frugiperda* neonate (<24 hour after hatching) was transferred using a fine painting brush into each well. Wells were covered with vented lids (CD International), and trays were held in an incubator at 27°C, 24-h scotophase, and 80% RH. Mortality and combined larval weights were recorded 7 d after infestation. Larvae that had not grown beyond first instar and weighed ≤ 0.1 mg were considered dead. Thus, mortality in this study includes both severe growth inhibition and death. Control mortality averaged 6% across treatments, and any replicates that exceeded 20% were not included. In each experiment, bioassays were replicated four times for each strain or cross, with 16 larvae per concentration (total of at least 64 larvae per concentration per cross).

Statistical Analysis

Concentration-mortality data were analyzed by probit analysis (Finney, 1971) using POLO-PC (LeOra Software 1987). LC_{50} and LC_{99} were calculated, together with their 95% confidence intervals, slopes and standard error. A likelihood ratio test was conducted to test that the LC_{50}'s were equal. Larval weights were transformed to percent growth inhibition relative to the controls and these data were analyzed by non-linear regression using PROC NLIN (SAS Institute 2011). Inverse regression was used to

estimate GIC_{50} (effective concentration at which 50% Growth Inhibition level is attained), their 95% confidence intervals, slopes and standard errors. The diagnostic concentration was determined based on the upper 95% confidence limit of the LC_{99} of the susceptible strain, then confirmed in separate bioassays with the susceptible and resistant strains (Marçon *et al*. 2000).

Sensitivity ratios were calculated using concentration-response statistics based on either mortality or growth inhibition. These values were calculated as the LC_{50} or GIC_{50} of the resistant strain divided by the LC_{50} or GIC_{50} of the susceptible strain (Robertson *et al.* 1995*,* 2007). When mortality was not generated and growth inhibition was no higher than 50% at the highest concentration used, the highest concentration was utilized to calculate the sensitivity ratio. Sensitivity ratios were regarded as equal if there was no overlap in the 95% confidence intervals (CI) of the estimate of these values.

Inheritance Experiments

To evaluate sex linkage and dominance of resistance, F_1 progeny from reciprocal crosses between resistant and susceptible strains (susceptible ♀ x resistant ♂ and susceptible ♂ x resistant ♀) were bioassayed as previously described and mortality curves were produced. Sex linkage was determined using hypothesis tests to compare the slopes and intercepts of probit regressions derived from reciprocal crosses and parental strains. We tested the null hypothesis that the lines are neither parallel nor equal using POLO-PC (LeOra Software 1987, Robertson *et al.* 2007). Dominance of resistance was calculated using the method of effective dominance at a fixed concentration:

$$D_X = (X_{RS} - X_{SS}) / (X_{RR} - X_{SS}),$$

where X_{SS}, X_{RS} and X_{RR} are the quantitative values calculated for a trait X for susceptible homozygotes, heterozygotes, and resistant homozygotes, respectively. Values of D_X range from zero or completely recessive resistance, to one representing completely dominant resistance. When D_X is 0.5, resistance is referred as codominant or additive (Bourguet *et al.* 2000). The traits used in the calculation of dominance were mortality (D_{ML}) and growth inhibition (D_{GIL}). D_{ML} and D_{GIL} were calculated at 7200 ng/cm^2. That rate was used in the calculations because it was the highest concentration tested, mortality and growth inhibition for the susceptible population were 100%, and there was no measureable effect on the resistant population. Estimates of D_{LC} (based on LC_{50}) could not reliably be assessed because mortality did not occur at the highest concentration in the resistant population and LC_{50} values could not be calculated (Bourguet *et al.* 2000, Storer *et al.* 2010).

To estimate the number of loci that confer resistance to Cry1F, F_1 progeny from reciprocal crosses were backcrossed to the resistant strain. The power of indirect tests for modes of inheritance is higher when the backcross progeny originate from crosses between the F_1 progeny and the parental strain most dissimilar in susceptibility (Roush and Daly 1990, Tabashnik 1991). The monogenic inheritance model was tested using a χ^2 test (Georghiou 1969, Preisler *et al.* 1990, Tabashnik 1991, Tabashnik *et al.* 1992). If resistance is monogenic, a backcross will produce progeny that are 50% RS and 50% RR. To test this hypothesis, the expected mortality in the backcross progeny at toxin concentration x was calculated using the formula

$$Y_x = 0.50\ (W_{RS} + W_{RR}),$$

where W_{RS} and W_{RR} are the mortalities of the presumed RS (F_1) and RR (parental line) genotypes at concentration *x* respectively interpolated from probit regression. A χ^2 goodness of fit test was conducted to determine differences between observed and expected mortality of the backcross response and expected response at each concentration (Tabashnik 1991; Tabashnik *et al.* 1992).

Cross-resistance

To determine if Cry1F resistance in *S. frugiperda* caused changes in susceptibility to other *Bt* toxins, the susceptible and resistant strains were bioassayed against Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry2Aa and Vip3Aa proteins. The same bioassay methodology described above was used for all bioassays. LC_{50}'s were calculated for Cry1Ab and Vip3Aa using POLO-PC (LeOra Software 1987). GIC_{50}'s were estimated for Cry1Ab, Cry1Ac and Vip3Aa with PROC NLIN (SAS Institute 2011). Sensitivity ratios were generated for these toxins as previously explained.

Both strains showed limited response to Cry1Aa, Cry1Ba and Cry2Aa. Therefore, the highest achievable concentration was used for bioassays and individual larval weights were recorded (64 larvae per strain). Larval weights were transformed to percent growth inhibition relative to the control, and an analysis of variance was used to identify significant differences in inhibition between susceptible and resistant strains using pairwise comparisons for each toxin (PROC GLIMMIX version 9.2.2, SAS Institute 2011).

Frequency of Resistant Alleles

An F_1 screen was used to identify the frequency of resistant alleles in field populations from areas where overwintering fall armyworm populations are known to occur (Texas and Florida). An F_1 screen involves crossing field-collected individuals of unknown genotype with individuals from a resistant laboratory strain. The offspring are then bioassayed to allow discrimination between resistant homozygotes, susceptible homozygotes and heterozygotes (Gould *et al.* 1997, Mahon *et al*. 2010).

S. frugiperda populations were collected in Florida and Texas in 2010 and 2011 (Table 1). Immature insects, eggs and larvae from the field were reared on artificial diet and allowed to pupate as previously described. Pupae were sexed and individually paired with one or two individuals of the opposite sex from the resistant laboratory strain. Adults were placed in "honeymoon cages" made of 27-gauge woven hardware cloth with a 33 mm diameter disposable plastic petri dish (Sterilin, Newport, UK) used as bottom and top. The cages were 4.2 cm tall. Each cage had an opening on the top where a cotton ball saturated with adult diet was placed. To prevent diet dehydration, water was added every day. Wax paper was placed around the cage to provide an oviposition substrate.

Eggs from each pair were collected daily and allowed to hatch. At least 48 neonates per pair were bioassayed with a Cry1F diagnostic concentration (200 ng/cm^2) as described previously. Pairs were considered successful when they produced enough hatched neonates to be tested.

The expected mortality at the diagnostic concentration is dependent on the genotype of the field-collected parent. If the field-collected parent is homozygous for susceptibility, the resulting progeny should all be heterozygotes resulting in 100%

mortality at the diagnostic concentration. However, if the field collected parent carries one resistant allele a 1:1 ratio of heterozygotes to resistant homozygotes will result and approximately 50% mortality at the diagnostic bioassays would be expected. If the parent is homozygous for resistance, all progeny will be resistant and 100% survival at the diagnostic bioassay is expected (Gould *et al.* 1997, Mahon *et al*. 2010). Larvae from the families that were identified from the F_1screen as being resistant were pooled, reared to adults and sib-mated. F_2 offspring from these families were tested with the diagnostic concentration to confirm the presence of resistant alleles (Gould *et al.* 1997).

Information from the F_1 screenings was used to estimate resistance allele frequencies ($E[P_R]$). For each collection the Bayesian methods described by Yue *et al*. (2008) were used to estimate frequencies and 95% credible intervals for these estimates were obtained from equation (15) of Andow and Alstad (1999). To calculate the probability of a false negative (P_{N0}) in an F_1 screen, equation (5) of Wenes *et al.* (2006) was used. Differences between Florida and Texas total frequency of resistant alleles was analyzed using Fisher's exact test.

To test the prevalence of resistant alleles in Puerto Rico, field collected fall armyworm eggs were obtained from Puerto Rico in 2010, 2011 and 2012 (Table 1). Eggs were allowed to hatch and neonates were used for bioassays with the diagnostic concentration (200 ng/cm^2). Frequency of resistant alleles was calculated using the Hardy-Weinberg frequency of homozygotes ($q^2=\sqrt{q}$), assuming that the genotypes are in Hardy-Weinberg proportions (Falconer and Mackay 1996). Proportion of survival and frequency of resistant alleles between years was analyzed using a χ^2 test for homogeneity.

Results

Resistance Levels

Bioassays revealed that the resistant laboratory strain displayed a high level of resistance to Cry1F. The LC_{50} for the susceptible strain was 24.86 ng/cm^2 and for the resistant strain was greater than 7,200 ng/cm^2 (Table 2), which was the highest concentration of Cry1F used in bioassays of this strain. This concentration was used to estimate the sensitivity ratio, indicating that the resistant population displays > 387 fold resistance to purified Cry1F (Table 2) and represents a conservative estimate of the sensitivity ratio. The diagnostic concentration was calculated to be 200 ng/cm^2 based on the upper 95% confidence limit of the LC_{99}. The GIC_{50} for the susceptible strain was 1.48 ng/cm^2 and for the resistant strain was again more than 7,200 ng/cm^2. The sensitivity ratio for growth inhibition was higher than 4,865-fold (Table 2).

Inheritance of Resistance

LC_{50}'s, GIC_{50}'s and sensitivity ratios of reciprocal crosses and backcrosses are presented in Table 2. Analyses of mortality curves from the reciprocal crosses indicated that resistance to Cry1F in *S. frugiperda* is recessive and autosomal (Figure 1). The hypothesis of slope equality for mortality between the reciprocal crosses indicated that the slopes and intercepts are identical ($\chi^2 = 5.33$; df = 2; $P > 0.05$), confirming that resistance is autosomal (Robertson *et al.* 2007). Dominance was also examined by comparing the mortality response curves of the F_1 generation with the most similar parental strain, in this case the susceptible strain. The test of equality showed no

differences between the slopes and intercepts ($\chi^2 = 9.02$; df = 4; $P > 0.05$), indicating that resistance to Cry1F is recessive. The calculations of D_{ML} and D_{GIL} generated values of 0, confirming that resistance is recessive (Table 3).

We also tested the monogenic versus polygenic inheritance model by backcrossing the F_1 generation with the resistant strain (RS x RR) and comparing the progeny's response to the parent strains. The response curve of the backcross showed a plateau at 50% mortality (Figure 2), suggesting a 1:1 ratio of RS and RR genotypes. The direct test for monogenic inheritance showed no significant deviation between observed and expected mortality at five of seven concentrations (Table 4). At 11 and 33 ng/cm^2, however, observed mortality was significantly lower than expected mortality (Table 4), probably generated by genetic variance in the backcross progeny compared with the parental strains and the F_1 (Tabashnik *et al*. 2002). Because most of the concentrations tested were non-significant, resistance to Cry1F in *S. frugiperda* appears to be monogenic.

Cross-resistance

Cry1Ab and Cry1Ac were the only Cry proteins that generated a measurable response that allowed comparisons of the susceptible and resistant strains. Cry1Ab was the only Cry toxin with sufficient mortality in the susceptible strain to calculate an LC_{50}. The LC_{50} for the susceptible strain was 37.46 ng/cm^2. In comparison, the resistant strain had no mortality at 6000 ng/cm^2. The GIC_{50} were 3.8 and 167.5 ng/cm^2 for the susceptible and resistant strain, respectively. Sensitivity ratios for Cry1Ab were >160.17 times for mortality and 44.08 for growth inhibition (Table 5). The hypothesis of equality

was rejected ($\chi^2 = 74.11$; df = 2; $P > 0.05$) while the hypothesis of parallelism was not ($\chi^2 = 1.27$; df = 1; $P > 0.05$), indicating that intercepts are different, but slopes are equal (Robertson *et al.* 2007). For Cry1Ac, the susceptible strain did not exhibit significant mortality at any of the concentrations tested, although significant grown inhibition was observed. The GIC_{50} for the susceptible strain was 112.02 ng/cm^2 and for the resistant strain >15,000 ng/cm^2, the highest concentration tested. The calculated sensitivity ratio for growth inhibition in Cry1Ac was >133.9 (Table 5). Equal slopes with different intercepts indicate that for Cry1Ab and Cry1Ac both strains (resistant and susceptible) have qualitatively identical, but quantitatively different mortality responses (Robertson *et al.* 2007). Results from Cry1Ab and Cry1Ac bioassays suggest a low level of cross-resistance relative to Cry1F in the resistant *S. frugiperda* from Puerto Rico.

Responses of the susceptible and resistant strains to Cry1Aa, Cry1Ba and Cry2Aa were low. The highest achievable concentration for each toxin was used to record growth inhibition of both colonies (Figure 3). A *t*-test indicated that there were no significant differences in the response of the susceptible and the resistant strains for Cry1Aa ($t = -0.64$; df = 1; $P = 0.52$) and Cry1Ba ($t = -0.16$; df = 1, $P = 0.87$). In contrast, the Cry1F resistant strains exhibited significantly higher growth inhibition when exposed to Cry2Aa ($t = -4.10$; df = 1; $P < 0.0001$). These results suggest that there is no cross-resistance withCry1Aa and Cry1Ba, although there could be a slight level of negative cross-resistance between Cry2Aa and Cry1F (Figure 3).

Vip3Aa bioassays resulted in a similar response from both strains. The susceptible strain exhibited an LC_{50} of 25.77 ng/cm^2 and the resistant strain of 34.38 ng/cm^2. GIC_{50}'s were 3.31 ng/cm^2 for the susceptible strain and 2.27 ng/cm^2 for the resistant strain (Table

5). The hypothesis of equality for mortality between the susceptible and the resistant strain indicated that slopes and intercepts are identical ($\chi^2 = 5.5$; df = 2; $P > 0.05$), suggesting that there is no cross-resistance between Vip3Aa and Cry1F.

Frequency of Resistant Alleles

F_1 results to detect frequency of Cry1F resistant alleles in populations of *S. frugiperda* from Florida and Texas are presented in Table 6. Resistant alleles were more frequent for Florida than for Texas in both years. Five heterozygotes were found in Palm Beach County, Florida in 2010 representing an estimated resistant allele frequency of 0.1229. In 2011 six heterozygotes and three homozygote resistant individuals were identified in Palm Beach County, Florida resulting in an estimated allele frequency of 0.2472. Two heterozygotes were found in Hendry County, Florida with a subsequent estimated allele frequency of 0.0531. Although these locations are only 70 miles apart, differences in the frequency of resistant alleles were found.

No resistant alleles were found in Texas in 2010. In 2011 one homozygote resistant was found in Hidalgo County, resulting in an estimated allele frequency of 0.0247. One heterozygote was found in Nueces County in 2011 resulting in an estimated allele frequency of 0.1056. There were no differences in the sex of the wild carrier of the resistant allele for both Florida and Texas populations (Table 6). Florida's total frequency of resistant alleles for 2010 and 2011 was 0.1322, while for Texas was 0.02, Fisher's exact test indicated significant differences between states ($P < 0.0001$) (Table 6). When control mortality is 10% and the total number of F_1 larvae entering the screen is 30, the

probability of finding a false negative for a line (P_{No}) was $1.6x10^{-8}$ suggesting a very high detection probability (Wenes *et al.* 2006).

Bioassays performed on insects from Puerto Rico maize in 2010, 2011, 2012 and 2013 indicated that proportion of survival and frequency of resistant alleles varied between years ($\chi^2 = 44.92$; $P < 0.0001$). Regardless of fluctuations between years, high levels of resistant alleles remained constant for three years (Table 7).

Discussion

The present study confirms the results reported by Storer *et al.* (2010) in which *S. frugiperda* populations from Puerto Rico were highly resistant to Cry1F compared to a laboratory-susceptible population. Initial genetic characterization of resistance indicated that resistance was autosomal with no maternal effects, and highly recessive (Storer *et al.* 2010). Our results also indicate that resistance is autosomal and highly recessive based on both growth inhibition and mortality response curves in diet bioassays. In addition, bioassays of progeny resulting from crosses of the resistant parental strain to heterozygotes indicate that resistance to Cry1F in *S. frugiperda* is conferred by a single locus, which has not been previously reported.

Cross-resistance experiments suggest that there is significant cross-resistance to Cry1Ab and Cry1Ac, although the level of resistance is much lower than observed for Cry1F. These results are important to assist in identifying possible mechanisms of resistance and to guide decisions on which toxins are compatible for pyramided events. Storer *et al.* (2010) reported similar results with Cry1Ac, but lower levels of cross-resistance with Cry1Ab.

The Cry1F resistance ratios based on mortality and growth inhibition found here differed from Storer *et al*. (2010), although similar trends were observed. Discrepancies in levels of cross resistance between the two studies might be due to differences in the methodology, origin of the Cry proteins and/or the populations tested. Populations used in this study originated from collections made at different times and locations compared to those used by Storer *et al*. (2010), and it is known that *S. frugiperda* response to Cry1A proteins is variable across geographies (Monnerat *et al*. 2006). Cross-resistance between Cry1F and Cry1Ac and Cry1Ab suggests that altered midgut receptors could be responsible for resistance to Cry1F in *S. frugiperda.* Receptor binding studies with *S. frugiperda* and other Lepidoptera suggest Cry1A proteins share a common binding site with Cry1F (Férre and Van Rie 2002, Hernández-Martínez *et al.* 2009, Luo *et al.* 1999).

Results of bioassays with Cry1Aa, Cry1Ba and Cry2Aa indicate that fall armyworm is generally insensitive to these proteins, although some growth inhibition was observed at high doses. Cry1Aa and Cry1Ba showed no significant differences between the resistant and susceptible strains, indicating that there is no cross-resistance between these toxins and Cry1F. Although susceptibility to Cry2Aa was significantly higher for the resistant strain, the difference was slight and the suggestion of negative cross-resistance is uncertain. Finally, Vip3Aa bioassays suggest that there is no cross-resistance between Cry1F and Vip3Aa. This result supports the binding experiments that suggest a lack of competitive binding between Cry1F and Vip3A (Sena *et al*. 2009). These results suggest the high potential of Vip3Aa to control Cry1F-resistant *S. frugiperda* and the potential for the two toxins to be deployed as pyramided toxins.

The nature of Cry1F resistance inheritance (i.e., autosomal, recessive and conferred by a single locus) provides an efficient tool to detect resistance alleles among field populations using an F_1 screening approach. Results of these tests suggest that the Cry1F resistance allele detected in both Florida and Texas is the same as that observed in Puerto Rico. Based on these results, the frequency of resistant alleles in Florida can be as high as 13%, but localized differences may exist The frequency of resistance among Texas populations was much lower, but still detectable (0.02). These results are consistent with gene flow studies where genetic exchange between Puerto Rico and Florida has been identified based on mitochondrial haplotype ratios but there is limited genetic exchange between Florida and Texas (Nagoshi *et al.* 2010, Nagoshi *et al.* 2012). Migration of resistant individuals from Puerto Rico to Florida might be playing an important role in the higher frequency of resistant alleles in southern Florida, but local selection may also be affecting frequency estimates.

Prior selection pressures from *Bt* foliar sprays, and/or local selection with Cry1F expressing maize may also be affecting the frequency of resistant alleles. Unfortunately, it is difficult to determine the amount of Cry1F expressing maize grown in southern Florida. Local differences in frequencies between counties in Florida may be a result of differences in selection pressures with some areas having a greater production of Cry1F expressing maize. Additional studies are necessary to ultimately define the factors influencing the differences in frequency of resistant alleles between Florida and Texas, and local differences that may exist in southern Florida.

Results from discriminating bioassays from insects collected from Juana Diaz, Puerto Rico during 2010, 2011 and 2012 are similar to those reported by Storer *et al*.

(2012) who also tested a collection from that municipality. Neither growth inhibition nor mortality reached 90% at the highest Cry1F concentrations tested. Our results with the diagnostic bioassay indicated the frequency of Cry1F resistance remains high although a low frequency of susceptible alleles may exist. The frequency of resistant alleles reported in this study may not reflect other local populations from Puerto Rico, where collections from Santa Isabel and Lajas populations exhibited a complete lack of response to Cry1F in 2010 and 2011, indicating the absence of susceptible alleles (Storer *et al.* 2012).

Cry1F resistance in *S. frugiperda* is similar to the Cry1F laboratory-selected *O. nubilalis* in that inheritance of Cry1F resistance is autosomal, recessive and conferred by a single locus (Pereira *et al*. 2007, 2008). However, cross-resistance results differ slightly in that *O. nubilalis* exhibited low levels of cross-resistance to Cry1Ac and lack of cross-resistance to Cry1Ab, while *S. frugiperda* exhibited cross-resistance to both Cry1Ab and Cry1Ac (Pereira *et al*. 2007, 2008). Similarly, it has been suggested that the frequency of Cry1F resistant alleles in midwestern U.S. *O. nubilalis* populations may be higher than anticipated, and may have already been present at relatively high frequencies prior to introduction of Cry1F-expressing plants (Siegfried personal communication). Higher frequencies of Cry1F resistant *O. nubilalis* and *S. frugiperda* may suggest that there is a low fitness cost associated with Cry1F resistance in the absence of selection. Pereira *et al*. (2009) reported that Cry1F resistant *O. nubilalis* are not significantly different from susceptible larvae of similar genetic background based on a number of parameters associated with reproductive fitness. A similar pattern might be occurring in *S. frugiperda* based on the relatively high frequencies observed in Florida and the stability of resistance in Puerto Rico over a period of 4 years in the absence of selection pressure. It is

important to investigate in detail the existence of fitness costs associated with resistance. Comparisons of fitness traits, such as developmental time, fecundity and longevity (Siegfried *et al.* 2001, Pereira *et al.* 2009, Crespo *et al.* 2010) in the susceptible and resistant strains, as well as the F_1 progeny, will provide valuable information for resistance management and mitigation. Preliminary results indicate that resistance to Cry1F in *S. frugiperda* is not associated with fitness cost (Chapter 3).

Our results suggest that there is a risk for the evolution of field resistance in *S. frugiperda* populations outside Puerto Rico. However, because resistance is recessive its evolution may be delayed by compliance with refuge recommendations (Gould 1998). The current use of 50% refuge in the southern United States combined with effective pyramided crops with multiple modes of action against *S. frugiperda* could help delay the spread of resistant alleles (Adamczyk and Mahaffey 2008, Storer *et al.* 2012). To date, there have been no reports of reduced effectiveness of Cry1F-expressing maize against *S. frugiperda* in Florida or Texas (Tabashnik *et al.* 2009, Hardke *et al.* 2011, Storer *et al.* 2012). Nonetheless, implementation of monitoring programs together with the investigation of reports of unexpected damage to Cry1F-expressing maize should be a priority. If reduction of product efficacy is linked to changes in allele frequency, actions should be taken to limit survival of resistant insects and slow or prevent their spread (Siegfried *et al.* 2007). The use of insecticides when populations are high could also help reducing the frequency of resistant alleles (Storer *et al.* 2012).

In order to have a better understanding of the evolution of resistance in *S. frugiperda* in Puerto Rico it is important to continue studying other aspects of the biology of this insect that could be affected by the presence of resistant alleles (e.g. fitness,

behavior, migration). Further studies will help us understand how resistance evolved in Puerto Rico and to predict future problems with this insect. Understanding field-resistance will assist the development of better risk assessments, improve predictions of resistance to *Bt* crops in other Lepidoptera and maximize the benefits of current and future generations of transgenic crops. Information derived from Cry1F resistant *S. frugiperda* from Puerto Rico can guide resistant management strategies for Latin America where this insect is an important pest of corn and cotton. Planned deployment of *Bt* crops in Latin America suggests the need for resistant management programs designed for tropical areas where crop production is year round and pest pressure is continuous.

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Tables

Table 1. Collections of *S. frugiperda* from Florida and Texas used for F_1 analysis and from Puerto Rico to evaluate sensitivity to Cry1F.

Pop code	County	Date of Collection
FL1	Palm Beach FL	May 2010
FL2	Palm Beach FL	April 2011
FL3	Hendry FL	April 2011
TX1	Lubbock TX	August 2010
TX2	Lubbock TX	November 2010
TX3	Hidalgo TX	May 2011
TX4	Hidalgo TX	August 2011
TX5	Nueces TX	November 2011
DMPR10	Juana Diaz PR	February 2010
DMPR11	Juana Diaz PR	February 2011
DMPR12	Juana Diaz PR	January 2012

Table 2. Concentration response to Cry1F of resistant (rr), susceptible (SS), reciprocal crosses and backcrosses of *S. frugiperda* to Cry1F protein overlaid on artificial diet, as measured by both mortality (LC_{50}) and growth inhibition (GIC_{50}).

Pop	No. Replicates [a]	Mortality				Growth Inhibition			
		Slope ± SE	LC_{50} [b] (95% CL)	Sensitivity Ratio [c]	χ^2	Slope ± SE	GIC_{50} [b] (95% CL)	Sensitivity Ratio [c]	*F*
SS	4	3.36 ± 0.43	24.9 (18.3-32.4)	-	6.82	1.27 ± 0.05	1.48 (1.33-1.65)	-	7523
rr	4	NC [d]	>7,200	>289.16	NC [d]	NC [d]	>7,200	>4,864.9	NC [d]
SS♂ x rr♀	4	2.38 ± 0.28	23.7 (16.3-32.6)	0.95	6.58	0.98 ± 0.06	1.28 (1.09-1.49)	0.87	5527.5
rr♂ x SS♀	4	1.99 ± 0.21	16.9 (10.7-24.8)	0.68	8.05	0.92 ± 0.07	0.77 (0.63-0.91)	0.52	6551.3
SS x rr Pooled	6	2.20 ± 0.22	20.5 (13.1-27.9)	0.82	9.82	0.95 ± 0.06	1.003 (0.86-1.15)	0.68	7822.4
rr x F_1 Pooled	10	0.81 ± 0.052	231.2 (94.1-926.3)	9.29	39.23	0.17 ± 0.05	18.95 (14.3-24.3)	12.80	320.1

[a] Each replicate consisted of 16 insects at each of the seven concentrations of Cry1F protein. [b] Nanograms of Cry1F/cm^2 diet. [c] LC_{50} or GIC_{50} relative to the susceptible strain. [d] NC, not calculated due to insufficient dose response.

Table 3. Effective dominance estimates (*D*) for the Cry1F resistance trait in *S. frugiperda* from Puerto Rico compared with laboratory susceptible population. Mortality and growth inhibition measured at 7,200 ng/cm^2.

Response	SS (%)	RR (%)	F_1 (RR x SS) (%)	Dominance
Mean mortality (SD)	100 (0)	15.63 (2.02)	100 (0)	$D_{ML} = 0$
Mean growth inhibition (SD)	100 (0)	9.52 (0.23)	100 (0)	$D_{GIL} = 0$

Table 4. Direct test for deviation between observed and expected mortality for a monogenic model (df = 1).

Concentration	Observed		Expected [a]		χ^2	*P*
(ng / cm^2)	Dead	Alive	Dead	Alive		
1.2	4	185	9	180	2.80	0.09
3.7	13	179	15	177	0.18	0.67
11	22	169	36	155	6.67	0.01[b]
33	56	135	74	117	6.93	0.009[b]
100	108	84	99	93	1.57	0.21
300	100	92	105	87	0.60	0.44
900	115	77	106	86	1.66	0.20

[a] Expected % mortality at each concentration x, calculated as:

$Y_x = 0.5$ (% mortality of F_1 at x + % mortality of R x S (pooled))

[b] Probability values indicating significant differences between the observed and expected mortality ($P < 0.05$).

Table 5. Comparative susceptibility of *S. frugiperda* susceptible and resistant strains to Cry1F, Cry1Ab, Cry1Ac and Vip3Aa.

Protein	Pop	No Replicates [a]	Mortality				Growth Inhibition			
			Slope ± SE	LC_{50} (95% CI) [b]	Sensitivity Ratio [c]	χ^2	Slope ± SE	GIC_{50} (95% CI) [b]	Sensitivity Ratio [c]	*F*
Cry1F	SS	4	3.36 ± 0.43	24.9 (18.3-32.4)	-	6.82	1.27 ± 0.05	1.48 (1.33-1.65)	-	7,523
	RR	4	NC [d]	>7,200	>289.16	NC[d]	NC[d]	>7,200	>4,864.86	NC[d]
Cry1Ab	SS	1[e]	1.8 ± 0.33	37.46 (0.7-17.58)	-	6.12	0.89 ± 0.15	3.8 (2.4- 5.79)	-	448.6
	RR	1[e]	NC[d]	>6000	>160.17	NC[d]	1.05 ± 0.27	167.5 (78.7– 360.3)	44.08	75.64
Cry1Ac	SS	1[e]	NC[d]	NC[d]	NC[d]	NC[d]	0.42 ± 0.06	112.02 (80.6-149.6)	-	413.7
	RR	1[e]	NC[d]	NC[d]	NC[d]	NC[d]	NC[d]	>15,000	>133.9	NC[d]
Vip3Aa	SS	3	3.23 ± 0.39	25.77 (21.24-31.35)	-	3.01	1.01 ± 0.13	3.31 (2.35-4.37)	-	1735.8
	RR	3	2.27 ± 0.30	34.38 (27.82-42.48)	1.33	1.94	1.44 ± 0.026	2.27 (2.18-2.36)	0.69	146,809

[a] Each replicate consisted of 16 insects at each of the seven concentrations of protein. [b] Nanograms of protein/cm^2 diet. [c] LC_{50} or GIC_{50} relative to the susceptible strain. [d] NC, not calculated due to insufficient dose response. [e] Data collected from individual weights of larvae.

Table 6. Frequency of Cry1F resistant alleles in *S. frugiperda* populations from Florida and Texas in 2010 and 2011.

Year	Location	Total Pairs	Family Lines Screened	Resistant Alleles		Sex of wild carrier	$E[P_R]$ [a] (95% CI)
				Sr	rr		
2010	Palm Beach, FL	46	24	5	0	3 ♀ / 2 ♂	0.1229 (0.0468 - 0.2035)
2011	Palm Beach, FL	44	28	6	3	5 ♀ / 4 ♂	0.2472 (0.1322 - 0.3053)
	Hendry, FL	57	27	2	0	2 ♂	0.0531 (0.0113 - 0.1175)
	Total	147	79	13	3	8 ♀ / 8 ♂	0.1322 (0.0799 - 0.1729) [b]
2010	Lubbock, TX	38	20	0	0	-	0.0000
	Lubbock, TX	20	3	0	0	-	0.0000
	Hidalgo, TX	109	39	1	0	1 ♀	0.0247 (0.0031 - 0.0658)
2011	Hidalgo, TX	101	23	0	0	-	0.0000
	Nueces, TX	36	13	0	1	1 ♂	0.1056 (0.0233 - 0.2141)
	Total	304	99	1	1	1 ♀ / 1 ♂	0.0200 (0.0055 - 0.0426) [b]

[a] Resistant allele frequency.

[b] Resistant allele frequency $E[P_R]$ in Florida is significantly different from Texas (Fisher's exact test, $P < 0.0001$).

Table 7. *S. frugiperda* populations from Juana Diaz, Puerto Rico tested for sensitivity to Cry1F protein on artificial diet tested in 2010, 2011 and 2012.

Year	Number of Insects Tested	Survival	Frequency of Resistant Alleles [a]
2010	48	35	0.854
2011	224	182	0.901
2012	1118	808	0.85
2013	671	574	0.925

[a] Frequency of resistant alleles was calculated using Hardy-Weinberg frequency of homozygotes ($q^2 = \sqrt{q}$).

Proportion of survival and frequency of *r* alleles did not vary significantly between years (χ^2 test for homogeneity = 44.92, $P < 0.0001$).

Figures

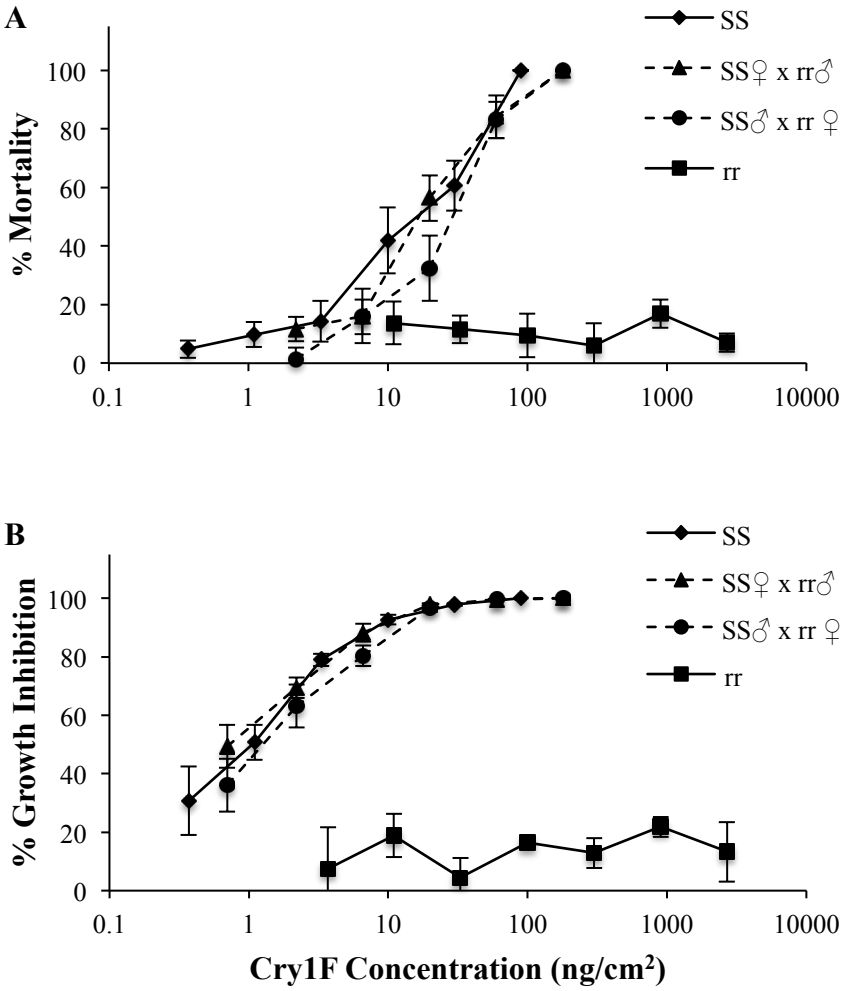

Figure 1. Concentration-response curves of susceptible (SS), resistant (rr) and progeny of reciprocal crosses of S. frugiperda to Cry1F protein. Each point represents mortality (A) and growth inhibition (B) observed in four replications (see Table 2) corrected for control mortality. Error bars represent standard error of the mean mortality or inhibition at each concentration.

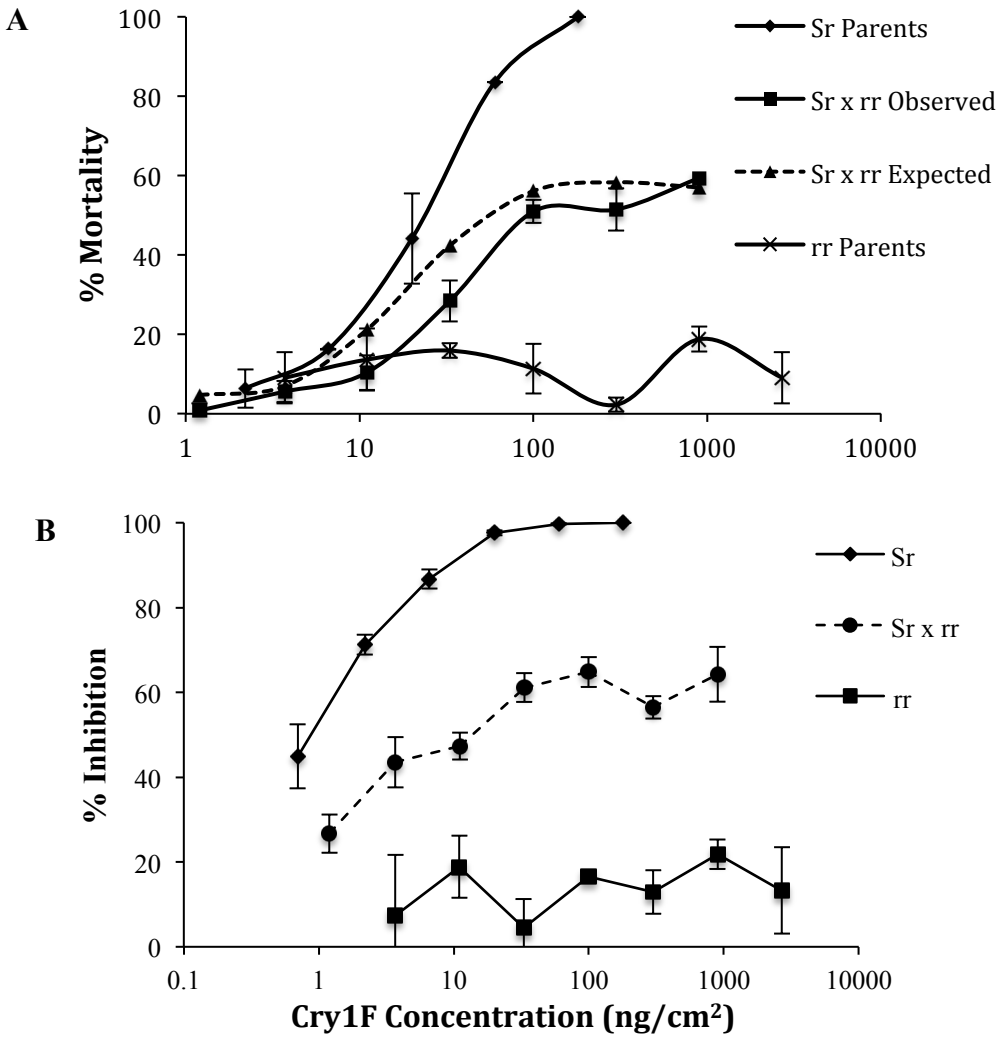

Figure 2. Concentration-response curves of the backcross progeny compared with those of the F_1 (rS) and the resistant parents (rr). Each point represents mortality (A) and growth inhibition (B) observed in four replications (see Table 2) corrected for control mortality. Error bars represent the standard errors of the mean mortality or inhibition at each concentration.

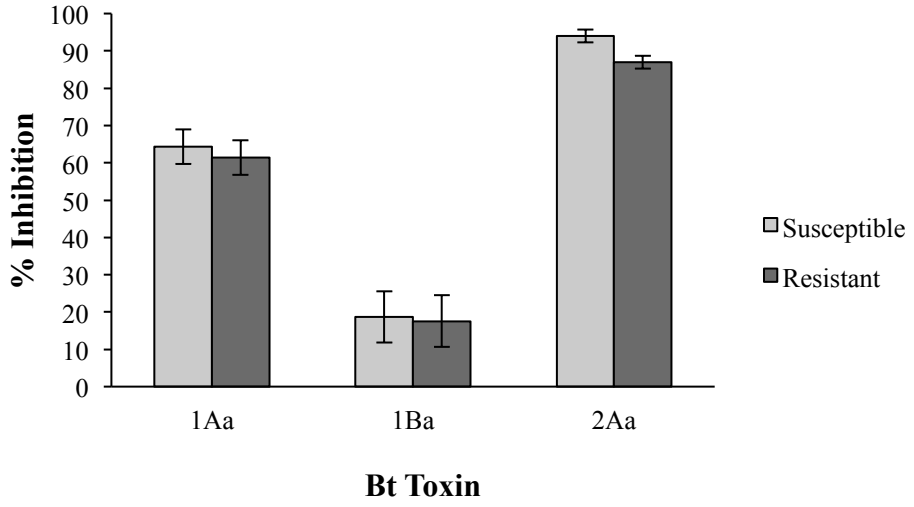

Figure 3. Percentage of inhibition produced by the highest dose of Cry1Aa (15,000 ng/cm^2), Cry1Ba (12,000 ng/cm^2) and Cry2Aa (5,000 ng/cm^2) in the susceptible and resistant strains. Error bars represent the standard error of the mean inhibition. Bars with the same letter are statistically similar (t test $P > 0.05$).

CHAPTER 3: Fitness costs of Cry1F resistance in fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

Introduction

Spodoptera frugiperda (J. E. Smith, 1797) is an important pest of corn, *Zea mays* L., in the Tropics and throughout the U.S. as a late season pest in late-planted crops (Buntin 1986, Mitchell *et al.* 1999). Conventional chemical control strategies are inconsistent and often unsatisfactory to control *S. frugiperda* in field corn (Siebert *et al.* 2008b). The most recent control tactic involves the use of transgenic maize expressing Cry1F from *Bacillus thuringiensis* Berliner. Maize hybrids containing Cry1F has been commercially available since 2003 and is marketed as Herculex® I *Insect Protection* (transformation event TC1507). Unexpected damage to Cry1F maize was reported in 2006 in Puerto Rico, and high levels of Cry1F resistance in *S. frugiperda* were reported (Matten *et a*l. 2008, Tabashnik *et al*. 2009). Storer *et al.* (2010) confirmed the high-level of resistance to Cry1F, and described inheritance of this resistance as autosomal and recessive. *S. frugiperda* resistance represents one of four species with documented field-evolved resistance to *Bt* crops. Resistance occurred after only four years of commercialization, making it the fastest documented case of field-evolved resistance to a *Bt* crop and the first case of resistance leading to voluntary withdrawal of a *Bt* crop from the marketplace (Tabashnik *et al*. 2009).

The study of fitness costs associated with resistance to *Bt* toxins is important for understanding resistance evolution and for evaluating resistance management practices that prevent or mitigate resistance to transgenic corn in the field (Carrière and Tabashnik

2001). Resistance alleles are rare during initial stages of resistance evolution, appearing almost entirely in heterozygotes (Georghiou and Taylor 1977). These newly arisen resistance traits are often assumed to be associated with fitness costs associated with resistance genes or with other loci closely linked to the resistance gene(s) (Gassmann *et al.* 2009). Individuals carrying a resistant allele will have a reduced fitness relative to susceptible individuals on non-*Bt* hosts (Ferré and Van Rie 2002). The relative fitness of heterozygotes influences response to selection and the rate of resistance evolution (Carrière and Tabashnik 2001). Models show that fitness costs can help delay resistance by selecting against *Bt*-resistant genotypes in refuges where insects are not exposed to *Bt* toxins (Tabashnik *et al*. 2003, Gassmann *et al.* 2009).

The pleiotropic effects of resistance alleles affect a variety of life history traits and can be seen as lower larval growth rate (Liu *et al*. 1999), survival (Groeters *et al.* 1994), or fecundity and mating success (Groeters *et al*. 1993, McKenzie 1996). The dominance of fitness costs of insect resistance to *B. thuringiensis* toxins have been assessed in several studies and in general, fitness costs associated with resistance have been recessive (Gassmann *et al.* 2009). Studies of fitness costs in absence of selection can be conducted in the laboratory either by monitoring the stability of resistance or by comparing one or more fitness components (e.g. survival, development rate, fecundity). Population cage studies provide a way of measuring the stability of resistance by observing changes in genotypic frequencies in a population comprised of mixed genotypes. These studies have the advantage of involving global measurement of fitness costs. However, population cage studies do not identify specific fitness components when an overall fitness cost has been determined. Therefore, both types of studies are important

because knowledge on specific fitness traits affected by resistance could improve measures to delay resistance (Roush and McKenzie 1987, Roush and Tabashnik 1990, McKenzie 1996, Gassmann *et al*. 2009). Whether single generation or population cage studies are used, comparisons must be made in a common genetic background. For extrapolation to the field, genotypes used in the experiments should be ideally field-derived. If estimates are not made in a common genetic background differences ascribed to resistance genotypes may be due to strain origin and associated epistatic interactions that are independent of relative fitness values at the resistance locus (McKenzie 1996).

Currently, no studies have addressed the fitness costs associated with Cry1F resistance in *S. frugiperda.* A susceptible and resistant strain with a similar genetic background, and their reciprocal crosses were used to study relative fitness linked to Cry1F resistance. Comparisons of life-history traits and population growth rates of the three genotypes (homozygous susceptible, heterozygous and homozygous resistant) were used to determine both the existence and dominance of fitness costs associated with resistance. Results are discussed in terms of their influence on the evolution of field-resistance in Puerto Rico and implications for resistance management.

Materials and Methods

Toxin source

Dupont Pioneer provided the Cry1F toxin used for selection (see Chapter 2 for details on protocols for toxin and quantification). Cry1F quantities were measured by SDS-PAGE/ densitometry (Crespo *et al*. 2008).

Insect Strains and development of new resistant strain for fitness comparisons

Dupont Pioneer (Johnston, IA) generated the Cry1F-selected strain, and the susceptible strain was purchased from BioServ (Frenchtown, NJ). Both strains were maintained using standard rearing techniques (Perkins 1979) with slight modifications (see Chapter 2 for details on protocols of insect strains and rearing). To minimize inbreeding or founder effects, and to insure a similar genetic background, the resistant strain was backcrossed twice with the susceptible strain. For each backcrossing event, fall armyworm pupae from the two strains were separated by sex based on morphological differences in genitalia (Heinrich 1919, Capinera 2000). Upon emergence, males and females were mass crossed with the opposite sex in two cages. Because resistance is recessive, autosomal and conferred by a single locus (Chapter 2), the F_1 progeny was randomly mated to obtain the F_2 progeny, which constituted rr, Sr and SS genotypes. The insects were then subjected to selection with a Cry1F diagnostic concentration (200 ng/cm^2) that allows only resistant homozygotes to survive (see Chapter 2 for details on bioassay protocol). The resistant survivors from this selection event were then subjected to a second cycle of backcrossing, random mating and selection. By the end of the second selection resistant and susceptible colonies were assumed to have 75% similarity in their genetic background.

Fitness comparisons were performed using four *S. frugiperda* genotypes: the Cry1F resistant strain (rr), the susceptible strain (SS), assumed homozygous for resistance and susceptibility, respectively, and the two F_1 progenies derived from

reciprocal crosses between the resistant and the susceptible strains (Sr). Reciprocal crosses were establishes as previously described.

Developmental time, pupal weight, and growth rate

Fitness comparisons were initiated with neonates of the fourth genotypes reared on multispecies lepidopteran diet (BioServ - Frenchtown, NJ). Neonates of each genotype were randomly selected from a pool of eggs laid on three different days to control for differences among sample cohorts (Robertson *et al*. 1995). On the first day, 64 neonates per cohort were individually transferred into 128-well bioassay trays (CD International, Pitman, NJ). After two days, the procedure was repeated for the second cohort and after another two days, a third cohort was established. A total of 64 larvae per cohort per genotype were tested representing 192 larvae per genotype. After seven days, larvae were weighed and transferred to 1 oz. translucent polystyrene soufflé portion cups (Solo Cup Company) with 4.5 ml of diet. Larvae were evaluated every day to record mortality and instar changes, which were determined by the presence of head capsules from the previous instar.

At pupation, individuals were weighed and sexed, then placed back in the cup until adult emergence, which was recorded daily. Larvae and pupae were maintained in an environmental chamber at 28°C ± 2°C and 68% ± 20% RH under a LD 16:8 h cycle.

Data collected from daily evaluations were used to calculate the developmental time and the growth rate (GR). Developmental time was estimated as the time in days from the moment the egg is laid to adult emergence. GR was calculated using the formula (Radford 1967):

$$GR = [W_2\ (mg) - W_1\ (mg)]/T$$

where W_1 and W_2 are the initial larval and pupal weights, and T is the time (days) from L_1 to the pupal stage (Sayyed and Wright 2001).

Fecundity and number of offspring produced

For each genotype, 18 newly emerged-adult males and females from the growth rate study were randomly paired in "honeymoon cages" representing six cages per cohort per genotype (see Chapter 2 for details on pair mating protocols). Wax paper sheets were inspected for eggs daily. Eggs were weighed and transferred to petri dishes (6 cm diameter) containing a moistened filter paper. Eggs were kept at room temperature to allow hatching. Most eggs hatched within five days, but hatching was recorded for a total of eight days. A minimum of ten neonates were transferred to 1 oz. cups with diet and reared until emergence to estimate the neonate-to-adult survivorship and the number of offspring female moths produced by each parental female (Pereira *et al*. 2009, Crespo *et al*. 2010).

To estimate the daily number of eggs and neonates produced per female, hatched neonates and unhatched eggs were preserved in 70% ethanol for posterior counts. Pictures of petri dishes with ethanol were taken using a Dino-Lite Pro digital microscope (Torrance, CA). ImageJ software (Schneider *et al*. 2012) cell counter function was used to count the number of neonates and unhatched eggs per female per day. Daily number of eggs per female was estimated by adding the number of neonates and unhatched eggs.

Male fitness was determined by the success and mating frequency and was measured by recording the number of spermatophores transferred. Females were frozen

and dissected under the microscope to extract spermatophores inside the bursa copulatrix. A successful mating was determined by the presence of spermatophores and the number of spermatophores found determined the number of times each pair had mated (Pereira *et al*. 2009, Crespo *et al*. 2010).

Population growth parameters

To estimate parameters related to the population growth potential in each *S. frugiperda* genotype, we assumed that the population has an exponential growth that is described by the model:

$$N_t = N_0 \, x \, e^{rm \, x \, t}$$

where N_t is the size of the population at time t, N_0 is the initial size of the population, and rm is a parameter related with the rate of population growth referred as the intrinsic rate of increase (Birch 1948). A life-table (Carey 1993, Southwood and Henderson 2000) was calculated to determine numerical differences among the parameters measured for resistant and susceptible homozygotes and heterozygotes from the two reciprocal crosses. Population growth parameters were determined as described by Birch (1948) and Carey (1993), and the SAS protocol explained by Maia *et al*. (2000) was used. The intrinsic rate of increase (*rm*) was calculated by an iteration of the Lotka equation:

$$\Sigma e^{-rx} l_x m_x = 1$$

where x is the pivotal age class, l_x is the survivorship at the pivotal age x, and m_x is the number of adult-females offspring produced by all experimental females alive at each pivotal age x (Birch 1948). The net reproductive rate, $R_0 = \Sigma (l_x m_x)$, the mean generation

time, $T = ln(R_0/r)$, and the finite rate of increase per day, $\lambda = ln\ (R)$ were also calculated (Pereira *et al*. 2009, Crespo *et al*. 2010).

Statistical analyses

Differences among pupal weight, developmental time, mean relative growth rate, number of eggs per female, number of neonates per female, egg weight per day, and number of spermatophores transferred were determined by analysis of variance (ANOVA) using PROC GLIMMIX (version 9.3; SAS Institute 2011). An initial analysis using studentized residuals was used to confirm the assumption of normality. When data were not normally distributed a log transformation was performed prior to analysis. A test for equality of covariance (covtest) was used to test for homogeneity of variance. When homogeneity of variance was not met, Tukey's adjustment was performed (Crespo *et al.* 2010). The means were separated at α=0.05 using least square means (PROC GLIMMIX) (SAS Institute 2011).

Variances associated with the population growth parameters were estimated by the jackknife method (Meyers *et al*. 1986) using the SAS program developed by Maia *et al*. (2000). The program allows the calculation of confidence intervals for all estimated parameters, as well as provides one-sided and two-sided *t*-tests to perform pairwise comparisons between groups, with their respective *P* values (Maia *et al*. 2000).

Population cage study

To test the stability of Cry1F resistance in *S. frugiperda,* two lines with a frequency of resistant alleles fixed at 0.5 were used. The two lines consisted of the

reciprocal crossed used to determine the developmental time previously described. Each cross was allowed to randomly mate for an additional generation to achieve Hardy-Weinberg equilibrium (Falconer and Mackay 1996). Hardy-Weinberg equilibrium was confirmed using bioassays with a Cry1F diagnostic concentration as previously described. Both crosses were tested for five additional generations to estimate changes in the frequency of resistant alleles. At each generation, eggs were taken at random and neonates bioassayed with the Cry1F diagnostic concentration with a minimum of 200 individuals per cross. Additionally, neonates from the resistant and susceptible strains were evaluated every generation to confirm the activity of the toxin.

For each generation and cross, resistant alleles frequencies were estimated using the Hardy-Weinberg frequency of homozygotes ($q^2=\sqrt{q}$), assuming that the genotypes are in Hardy-Weinberg proportions (Falconer and Mackay 1996). The 95% confidence intervals were obtained using the normal approximation to binomial probabilities (Sokal and Rohlf 2012). To calculate the probability of a false negative (P_{N0}), equation (5) of Wenes *et al.* (2006) was used. The frequencies of resistant alleles were analyzed with a linear regression using PROC REG and the Pearson correlation coefficients were obtained through PROC CORR (SAS Institute 2011).

Results

Development time, pupal weight, and growth rate

Significant differences ($P < 0.05$) were found among genotypes and cohorts for developmental time, pupal weight, and growth rate. Additionally, significant differences

were found between sexes for both pupal weight and developmental time, but not for growth rate. Interactions between genotypes and cohort were significant in all life history traits, while thegenotype by sex interaction was not significant for any of the parameters measured (Table 1).

Pupal weight, developmental time and growth rate comparisons were made by sex across genotypes (Table 2). Overall, significant differences ($P < 0.05$) were found between heterozygotes that originated from susceptible males and resistant females (i.e. S♂r♀) and the rest of the genotypes. Pupal weight was the only factor that showed significant differences between females and males, on average males had higher weight than females. In addition, pupal weights of S♂r♀ males and females were significantly higher than the rest of the genotypes and pupal weights of homozygous susceptible females were significantly lower than homozygous resistant females. Larval developmental time in S♂r♀ was significantly lower than the susceptible and resistant genotypes. Males' developmental time was significantly longer for the resistant strain relative to the susceptible and the reciprocal crosses. However, the magnitude of difference between susceptible and resistant homozygous males was approximately one day. Growth rate differences displayed a similar trend in both sexes and S♂r♀ had a higher growth rate relative to S♀r♂, susceptible and resistant.

Pupal weights and growth rates suggest that there is a slight indication of hybrid vigor, because differences between genotypes in all life history traits were probably affected by the differences in the cohorts. Heterozygotes originated from S♂r♀ always had significant differences between cohorts in the majority of the comparisons, while the rest of the genotypes exhibited significant differences in a few of the comparisons. No

clear developmental effect was associated with resistance and no indication that the resistance alleles were associated with a fitness cost.

Fecundity and number of offspring produced

Figure 1 shows the average number of eggs produced by female, the egg weight per day per female, and the number of hatched neonates per female from four *S. frugiperda* genotypes tested. Among the *S. frugiperda* genotypes tested, there were no significant differences in the number of eggs produced by females ($F = 0.25$; df = 3, 59; $P = 0.8624$), weight of eggs produced per day ($F = 1.46$; df =3, 586; $P = 0.2251$) and number of offspring ($F = 0.58$; df = 3, 59; $P = 0.6304$). No significant differences were found in the number of eggs and number of hatched neonates per female. However, the egg weight per day per female was significantly higher for S♂r♀ compared to homozygous susceptible.

Figure 2 shows the number of spermatophores obtained from the 18 females tested per each genotype. There were no significant differences in the mean number of spermatophores ($F = 0.61$; df = 3, 59; $P = 0.6139$) transferred to females among the four *S. frugiperda* genotypes tested.

Population growth parameters

Table 3 summarizes five life table statistics for all *S. frugiperda* genotypes compared. The net reproductive rate (R_0), the intrinsic rate of increase (r_m), the doubling time (DT) and the finite rate of population increase (λ) did not differ significantly

between the four genotypes tested. The mean length per generation (*T*) was significantly higher for S♀r♂ than for S♂r♀ and the homozygote genotypes; however the difference was of less than a day.

Population cage study

Table 4 summarizes the frequency of resistant alleles tested for seven generations in two strains derived from reciprocal crosses of resistant and susceptible *S. frugiperda*. When control mortality is 10% and the total number of larvae entering each screen is >300, the probability of finding a false negative for a line (P_{No}) was $< 1.6 \times 10^{-8}$ suggesting a very high detection probability (Wenes *et al.* 2006). The frequency of resistant alleles decreased after seven generations in both crosses (Figure 4) although there were some generations when the frequency increased. The predicted equation from the linear regression analysis is represented in Figure 4. The correlation between predicted and observed frequencies was 0.77, indicating that the prediction equation was a good representation for observed values.

Discussion

Results from comparisons of fitness parameters indicate that there is not a major fitness cost associated with Cry1F resistance in either heterozygotes or homozygote resistant *S. frugiperda*. Significant differences were reported for a few parameters. However, differences were mainly between homozygous genotypes (i.e. susceptible and resistant) with heterozygotes. Such differences were minor with no indication of a fitness

cost for the homozygote resistant insects as the heterozygous individuals were equally fit or fitter that the susceptible homozygotes. Such differences suggest the presence of hybrid vigor which may be an artifact of genetic divergence of the strains, however, it cannot be ruled out that the genetic differences contributed to the effects observed in some fitness parameters (Gassmann *et al.* 2009). Results from heterozygotes also suggest that if there is a fitness cost that was not detected, it is likely to be recessive (Pereira *et al*. 2009).

Results indicate that resistance to Cry1F in *S. frugiperda* and *O. nubilalis* is not just similarly inherited (Chapter 2), but it also involves the lack of fitness cost for both species (Pereira *et al*. 2008, 2009). Elucidation of the resistance mechanism in *S. frugiperda* is necessary to identify the resistance-associated mutation. Fitness costs are better understood when mutations that confer resistance have been identified, allowing a better understanding of the physiological consequences of resistance (Coustau *et al*. 2000). It has been hypothesized that cadherin mutations conferring resistance to *Bt* toxins cause fitness costs by increasing permeability of the gut membrane (Gassmann *et al*. 2009). However, resistance to Cry1F in European corn borer has not been shown to involve reduced toxin binding to midgut epithelia or enhanced degradation by luminal gut proteases (Pereira *et al*. 2009). Similarly, Cry1F resistance in *S. frugip*erda might not be conferred by a cadherin mutation, but by some other mechanism similar to *O. nubilalis*. The mechanism of resistance to Cry1F in *S. frugiperda* has yet to be determined. Further studies that identify the Cry1F resistance mechanism in *S. frugiperda* will help us understand the physiological consequences of resistance and to better understand the apparent lack of major fitness costs (Coustau *et al*. 2000).

The present study represents the first step toward understanding fitness costs associated with Cry1F resistance in *S. frugiperda*. It should be noted that the estimates of relative fitness were made with insects reared on artificial diet and under optimized environmental conditions for *S. frugiperda* rearing. Fitness of *S. frugiperda* in the laboratory might differ in their fitness under natural conditions or in unfavorable environments (Janmaat and Myers 2005, Raymond *et al*. 2005). If fitness costs are magnified by environmental stresses experienced in the field but not in laboratories, fitness costs may be underestimated (Gassmann *et al*. 2009). Usually, fitness costs are greater on plants than in diets (Gassmann *et al.* 2009, Orr 2009). Additionally, fitness might vary spatially, and within a generation a genotype might enjoy higher fitness if it resides in one region but lower fitness if it resides in other regions (Orr 2009). Further experiments on plants under greenhouse conditions that emulate field settings with different ecological conditions are needed to corroborate the lack of fitness cost associated with Cry1F resistance in *S. frugiperda* (Gassmann *et al.* 2009, Orr 2009). It is also important to perform experiments to determine the fitness differences of resistant insects developing in Cry1F corn and the respective isoline to determine the impact of insects emerging in *Bt* corn compared to those emerging in refuges (Gassmann *et al*. 2009). The combination of several approaches should provide a most robust estimate of relative fitness of individuals carrying resistant alleles (Gassmann *et al.* 2009, Orr 2009). Likewise, documentation of fitness cost influencing mating competition, pheromone response, first-male paternity and flight capacity may provide valuable insight into trade-offs between resistance and fitness (Groeters *et al*. 1993).

Results of the population cage study indicated a decrease in the frequency of resistant alleles after seven generations. However, it is uncertain whether this decline represents a fitness cost that was undetected in the previous experiments or whether this decrease is the result of random drift. The effects of fitness on mating behavior may also be influencing the decrease in resistant alleles. Fitness costs associated with mating behavior were reported in *Plutella xylostela* resistant to a *B. thuringiensis* formulation. In this case resistant males had a lower mating success than susceptible males (Groeters *et al*., 1993). Additionally, with the low number of replications (crosses) is difficult to conclude the exact cause of the decrease in the frequency of Cry1F resistant alleles in *S. frugiperda*. Future studies assessing mating behavior differences between genotypes and cage studies including more replications should be considered.

Results of this study have important implications to resistance management. Equivalent fitness of heterozygotes and susceptible homozygotes and no fitness cost in resistant homozygotes may contribute to the persistence of resistant alleles (Roush and McKenzie, 1987) among field populations. Our findings are consistent with the data from field-collected insects from Puerto Rico (Chapter 2) where resistance frequencies have remained high although Cry1F maize was commercially withdrawn in the island in 2007 (Storer *et al*. 2012). The lack of fitness cost may also have contributed to the higher than expected resistant allele frequency in Florida and Texas, and could have had an influence in the initial frequency of resistant alleles prior to selection. The lack of fitness cost in Cry1F resistant *S. frugiperda* might affect the durability of Cry1F corn and make remediation and management tactics more challenging.

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Tables

Table 1. *F*-test values and probabilities for the three-factor analysis of variance on life-history traits of larvae from four *S. frugiperda* genotypes reared on artificial diet.

Source	df	Pupal weight		Developmental time		Growth rate [a]	
		F	*P*	*F*	*P*	*F*	*P*
Genotype	3	11.77	<0.0001	15.53	<0.0001	22.89	<0.0001
Sex	1	41.04	<0.0001	20.36	<0.0001	2.44	0.1190
Cohort	2	21.69	<0.0001	32.0	<0.0001	47.87	<0.0001
Genotype x Sex	3	1.16	0.3239	1.08	0.3563	2.02	0.1096
Genotype x Cohort	6	9.56	<0.0001	7.70	<0.0001	13.81	<0.0001
Sex x Cohort	2	5.63	0.0038	0.72	0.4881	3.20	0.0416
Gen. x Sex x Co	6	1.71	0.1164	1.64	0.1338	1.96	0.0697
Error	608						

Measurements were obtained from 68 to 99 individuals

[a] Growth rate = (final larval mass – initial larval mass) / number of days (see Material and Methods)

Table 2. Comparison of fitness components of larvae (mean ± SE) from fourth *S. frugiperda* genotypes reared on artificial diet (n = 68 - 99).

Strain or Cross	Fitness Components					
	Pupal weight (mg)		Developmental time (days)		Growth rate (mg/day)	
	♂	♀	♂	♀	♂	♀
SS	294.5 ± 3.3 [b]	274.8 ± 3.4 [d]	28.6 ± 0.3 [b]	27.8 ± 0.3 [c]	10.4 ± 0.2 [cd]	10.0 ± 0.2 [d]
S♀r♂	300.8 ± 3.4 [ab]	281.6 ± 3.2 [cd]	28.0 ± 0.3 [bc]	27.7 ± 0.3 [c]	10.8 ± 0.2 [bc]	10.3 ± 0.2 [d]
S♂r♀	308.5 ± 3.0 [a]	298.7 ± 3.4 [b]	27.6 ± 0.2 [c]	26.7 ± 0.3 [d]	11.2 ± 0.1 [ab]	11.3 ± 0.2 [a]
rr	296.7 ± 3.6 [b]	284.8 ± 3.4 [c]	29.5 ± 0.3 [a]	28.3 ± 0.3 [bc]	10.1 ± 0.2 [d]	10.2 ± 0.2 [d]

For each fitness component, means followed by the same letter are not significantly different (*t* LSD test, $P > 0.05$).

Growth rate = (final mass – initial mass) / number of days (see Materials and Methods)

Table 3. Comparison of population growth parameters mean (95% confidence intervals) for *S. frugiperda* genotypes reared on artificial diet (n = 17 - 18).

Strain or Cross	Population growth parameter				
	R_0	r_m	T	DT	λ
SS	584.4 [a]	0.25 [a]	25.43 [a]	2.74 [a]	1.29 [a]
	(360.4 – 808.5)	(0.23 – 0.27)	(25.07 – 25.78)	(2.53 – 2.97)	(1.26 – 1.31)
S♀r♂	714.97 [a]	0.25 [a]	26.14 [b]	2.74 [a]	1.29 [a]
	(473.2 – 956.8)	(0.24 -0.27)	(25.78 – 26.49)	(2.57 – 2.91)	(1.27 – 1.31)
S♂r♀	604.2 [a]	0.25 [a]	25.87 [ab]	2.79 [a]	1.28 [a]
	(454.1 – 754.3)	(0.24 – 0.26)	(25.36 – 26.38)	(2.69 – 2.9)	(1.27 – 1.29)
rr	674.5 [a]	0.26 [a]	25.46 [a]	2.71 [a]	1.29 [a]
	(579.7 – 769.3)	(0.25 – 0.26)	(24.99 – 25.93)	(2.64 – 2.78)	(1.28 – 1.3)

R_0, net reproductive rate (females/female/generation); r_m, intrinsic rate of population increase (per day); T, mean length of a generation (days); DT, time for the population to double its size (days); λ, finite rate of population increase (per day) (see Materials and Methods) (Maia *et al*. 2000). For each parameter, values followed by the same letter are not significantly different for two-tailed *t*-tests for pairwise group comparisons ($P > 0.05$).

Table 4. Frequency of resistance alleles from *S. frugiperda* reciprocal crosses between Cry1F resistant and susceptible strains during seven generations.

Generation	Cross	N	Survival	r Frequency [a]	95% CI
F_1	SS♀ x rr♂	224	0	0.5	-
	SS♂ x rr♀	224	0	0.5	-
F_2	SS♀ x rr♂	663	160	0.492	(0.45 – 0.53)
	SS♂ x rr♀	672	132	0.4442	(0.40 – 0.48)
F_3	SS♀ x rr♂	582	113	0.4418	(0.40 – 0.48)
	SS♂ x rr♀	540	125	0.4822	(0.44 – 0.52)
F_4	SS♀ x rr♂	548	102	0.4328	(0.39 – 0.47)
	SS♂ x rr♀	551	105	0.4378	(0.40 – 0.48)
F_5	SS♀ x rr♂	448	50	0.3367	(0.29 – 0. 38)
	SS♂ x rr♀	448	46	0.3232	(0.28 – 0.37)
F_6	SS♀ x rr♂	448	56	0.3559	(0.31 – 0.40)
	SS♂ x rr♀	432	98	0.4776	(0.43 – 0.53)
F_7	SS♀ x rr♂	557	73	0.3638	(0.32 – 0.40)
	SS♂ x rr♀	445	48	0.3311	(0.29 – 0.38)

[a] Frequency of resistant alleles was calculated using Hardy-Weinberg frequency of homozygotes ($q^2 = \sqrt{q}$).

Figures

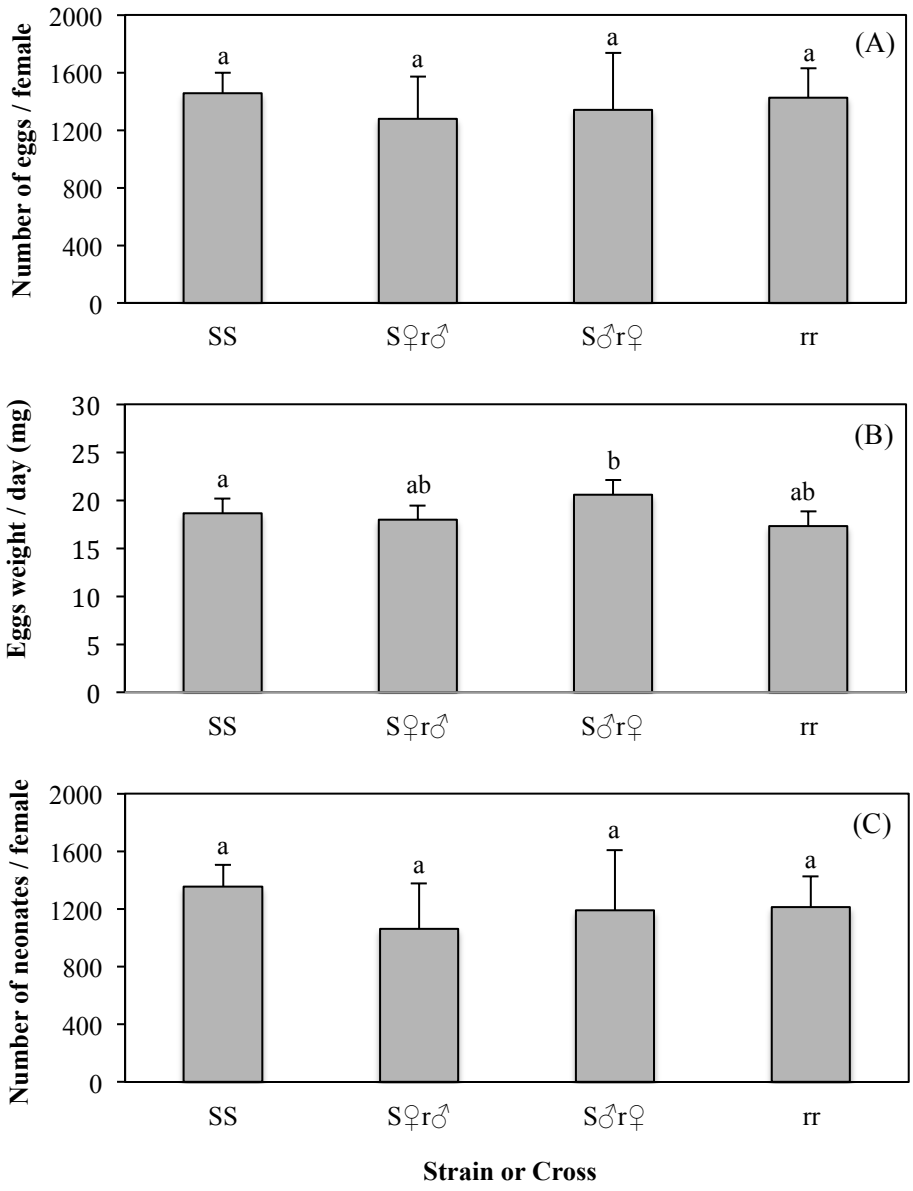

Figure 1. Comparison of fecundity parameters among *S. frugiperda* genotypes. (A) Number of eggs per female (n = 17-18), (B) eggs weight per day (n = 143-158), (C) number of neonates per female (n = 17-18). Error bars represent standard errors. Bars followed by the same letter are not significantly different (*t* LSD test, $P > 0.05$).

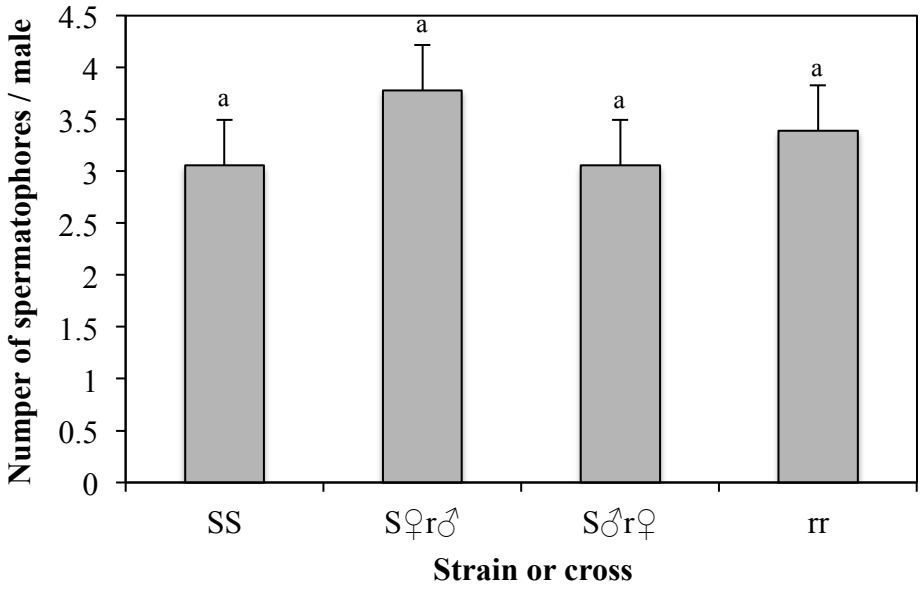

Figure 2. Comparison of the number of spermatophores produced per male males among *S. frugiperda* genotypes (n = 18). Bars followed by the same letter are not significantly different (*t* LSD test, *P* > 0.05).

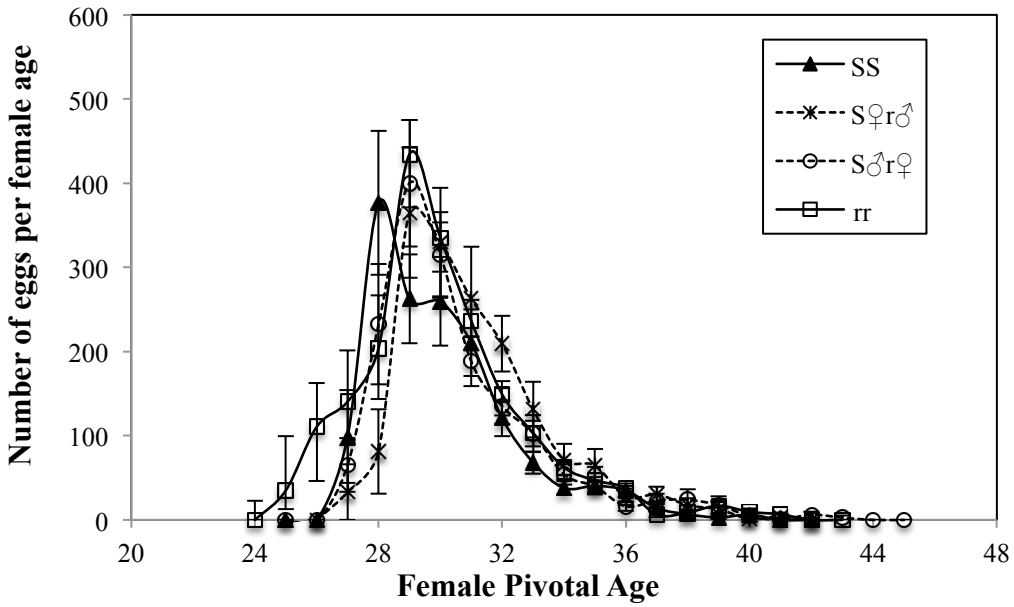

Figure 3. Egg production patterns of females from fourth *S. frugiperda* genotypes (n = 17-18).

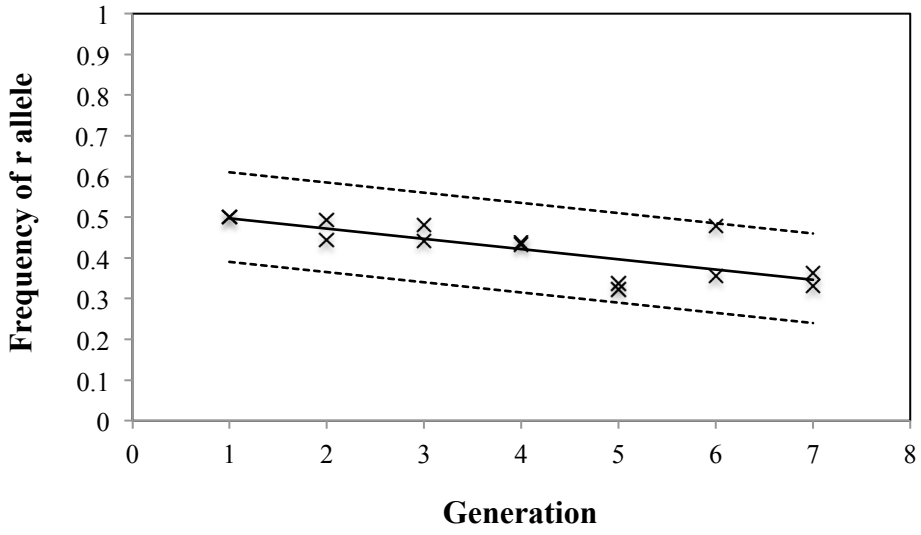

Figure 4. Linear regression of the frequency of resistant alleles from seven generations of two strains derived from crosses between Cry1F resistant and susceptible *S. frugiperda.* The correlation between observed and predicted frequencies was 0.77. Dashed lines indicate the 95% predicted limits.

CHAPTER 4: Effect of Cry1F corn on larval feeding and movement of susceptible and Cry1F resistant *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae)

Introduction

To delay the evolution of pest resistance to *Bacillus thuringiensis* (*Bt*) crops, the Environmental Protection Agency (EPA 2001) has mandated the high-dose/refuge strategy (HDR). This strategy requires farmers to plant refuges of non-*Bt* crops to promote the survival of susceptible insects. Refuges allow susceptible insects to survive and to far outnumber the few resistant individuals that survive in *Bt* crop fields, thereby reducing the probability that resistant insects will mate with each other. If resistance is recessive, mating between susceptible and resistant adults will produce offspring that cannot survive on *Bt* plants because of the high-dose expression. Thus, the frequency of resistant individuals will be greatly reduced. To facilitate mating between resistant and susceptible insects, variety of refuge strategies have been considered. These strategies include planting refuges in blocks, strips or seed mixtures (Bates *et al*. 2005).

Onstad *et al*. (2011) suggest that neither blocks nor mixtures are clearly superior. However, mixtures will make pest monitoring more difficult, lead to greater *Bt* corn adoption by farmers, and may increase the risk of resistance because of larval movement between expressing and non-expressing plants. Conversely, block refuges have the disadvantage of the concern of IRM compliance by farmers (Onstad *et al*. 2011). Current Lepidoptera Insect Resistance Management (IRM) strategies mainly involve blocks and in-field strips for events expressing one-toxin (EPA 2001, Onstad *et al*. 2011). However,

for some traits, the use of seed mixtures for IRM is being considered as a refuge option. The U.S. Environmental Protection Agency has approved the registration of a seed mixture for some pyramided events with activity against Lepidoptera (EPA 2010). However, each species must be considered independently and IRM practices should not be expected to be suited for all species (EPA 1998; Onstad *et al*. 2011). Thus, specific information about the behavior of different pests is important to appropriately define the details of resistance management plans. Particularly, the study of adult and larval movement is important to better estimate the durability of different refuge configurations. For example, studies indicating Lepidoptera larval movement from plant to plant define current strategies (blocks or in-field strips) for events expressing one toxin (Ross and Ostlie 1990). Movement may expose larvae to a lower dose of *Bt* toxins increasing the likelihood of heterozygote survival and potentially accelerating the evolution of resistance (Mallet and Porter 1992, EPA 1998).

Behavioral studies of lepidopteran larvae have shown that exposure to toxins present in *Bt* corn seems to increase the likelihood of larvae moving between plants, which may hasten resistance evolution. This behavioral response has been reported in a number of different lepidopteran species feeding on *Bt* plants including *Ostrinia nubilalis* (Hübner) (European corn borer) (Davis and Coleman 1997, Davis and Onstad 2000), *Plutella xyllostela* (L.) (diamondback moth) (Ramachandran *et al.* 1998), *Heliothis virescens* (F.) (tobacco budworm) (Parker and Lutrell 1999), and *Tricoplusia ni* (Hübner) (cabbage looper) (Li *et al*. 2006). Similar results were found in *Spodoptera exigua* (Hübner) (beet armyworm) (Berdegué *et al*. 1996), *O. nubilalis* (Davis and Coleman

1997), and *Epiphyas postvittana* (Walker) (light brown apple moth) (Harris *et al.* 1997) exposed to Cry toxins in the laboratory.

Fewer studies have examined the potential for resistance to interact with behavioral responses to *Bt* toxins (Prasifka, *et al*. 2009, 2010). Studies of larvae on diet containing Cry toxins have shown that movement is similar between resistant and susceptible strains. Resistant and susceptible strains of *H. viriscens* (Gould and Anderson 1991), *E. postvittana* (Harris *et al.* 1997), *S. exigua* (Berdegué *et al*. 1996), and *O. nubilalis* (Huang *et al*. 2001) tended to avoid diet containing *Bt* toxins. Additionally, *E. postvittana* (Harris *et al.* 1997) and *S. exigua* (Berdegué et al. 1996) exhibited increased movement after exposure to *Bt* toxin. In contrast, experiments with Cry1Ab resistant *O. nubilalis* indicated that resistant larvae did not avoid dietary Cry1Ab, but were more likely to be found on diet with *Bt* toxin and showed reduced movement when exposed to Cry1Ab diet compared to susceptible larvae (Prasifka *et al*. 2009, 2010). *O. nubilalis* video-tracking observations predicted that resistant larvae are more likely to disperse onto adjacent non-*Bt* plants, although such behavior may reflect greater survival after toxin exposure for resistant larvae rather than increased activity (Prasifka *et al.* 2010).

Spodoptera frugiperda (J. E. Smith, 1797) is an important pest of maize, *Zea mays* L., in the Tropics and throughout the U.S. as a late season pest in late-planted crops (Buntin 1986, Mitchell *et al.* 1999). *S. frugiperda* has been controlled with maize hybrids containing Cry1F since 2003. However, unexpected damage to Cry1F maize was reported in 2006 in Puerto Rico, which was associated with high levels of Cry1F resistance in *S. frugiperda* determined with diet bioassays (Matten *et al.* 2008; Tabashnik *et al*. 2009). Resistance was described as autosomal, recessive (Storer *et al*. 2010,

Chapter 2) and monogenic (Chapter 2). Studies regarding the behavior of susceptible and/or resistant *S. frugiperda* larvae have not been performed.

Cry1F resistance in *O. nubilalis* has not been reported in the field, but a laboratory-selected strain has been previously generated and characterized (Pereira *et al.* 2008b). Cry1F resistance in *S. frugiperda* and *O. nubilalis* has been identified as recessive, autosomal and conferred by a single locus (Pereira *et al.* 2008, Storer *et al.* 2010, Chapter 2). The availability of resistant strains from both species allowed addressing the effect of exposure to Cry1F on larval movement. Therefore, both species were used to conduct choice and no-choice experiments to investigate the possible effect of Cry1F resistance on the behavior of larvae. Neonates from Cry1F resistant and susceptible *S. frugiperda* and *O. nubilalis* strains were observed to determine whether resistance influences response to Cry1F. Phenotypes were confirmed using bioassays on artificial diet with a Cry1F diagnostic concentration. In addition, experiments were conducted to determine if irritability as a result of exposure to Cry1F expressing plant tissue influences movement. Results are discussed in terms of their implications for resistance management.

Materials and Methods

Insect Strains and Plant Material

Dupont Pioneer (Johnston, IA) generated the Cry1F-selected *S. frugiperda* strain (See Chapter 2 for details), and the susceptible strain was purchased from BioServ (Frenchtown, NJ). Both strains were maintained using standard rearing techniques

(Perkins 1979) with slight modifications (see Chapter 2 for details). The resistant *O. nubilalis* strain originated from insects collected throughout the central U.S. Corn Belt in 1996 and has been maintained in the laboratory (Pereira *et al*. 2008a, 2008b) with repeated exposure to a diagnostic concentration of Cry1F that allows only resistant homozygous individuals to survive (Pereira *et al.* 2008a). The susceptible *O. nubilalis* bivoltine E strain was generated in 1985 from field-collected insects near Geneva, NY and was augmented with additional field collections in 1996. *O. nubilalis* strains were maintained using standard rearing techniques (Lewis and Lynch 1969) with slight modifications (Siqueira *et al*. 2004).

Resistant and susceptible phenotypes were compared in each experiment. The susceptible *S. frugiperda* consisted of the F_1 progeny of crosses of the parental susceptible and resistant strains, and the *O. nubilalis* susceptible phenotype consisted of a mixture of homozygous susceptible with a low frequency of heterozygous individuals. Cry1F resistance in both species has been characterized as completely recessive, autosomal and conferred by a single locus; therefore heterozygous individuals are considered functionally susceptible (Pereira *et al*. 2008a, Chapter 2). Resistant and susceptible phenotypes were used because the susceptible *S. frugiperda* strain exhibited significant behavioral differences compared to the resistant strain probably as a result of its continuous rearing on artificial diet. Crossing homozygous susceptible and resistant insects generated heterozygous *S. frugiperda*; for this purpose pupae from the resistant and susceptible strains were separated by sex based on morphological differences in genitalia and upon emergence (Heinrich 1919, Capinera 2000), virgin males and females were mass crossed with the opposite sex from each strain.

Bioassays on artificial diet were performed before each experiment to confirm susceptible and resistant phenotypes (see Chapter 2 for details). Neonates of each phenotype were bioassayed with a Cry1F diagnostic concentration of 200 ng/cm^2 for *S. frugiperda* and 60 ng/cm^2 for *O. nubilalis*. Dupont Pioneer provided the Cry1F toxin used for selection (see Chapter 2 for details on toxin production). Cry1F concentrations were confirmed by SDS-PAGE/densitometry (Crespo *et al*. 2008). After the phenotypes were confirmed, neonates within 12 hours of eclosion were used for all experiments.

Plant Material

Leaf discs of TC1507 corn that express Cry1F and the respective isoline were used to identify behavioral responses. Plants were grown in the greenhouse up to V7 and used between V7 to V9. Cry1F expression was confirmed using Bt1F trait check lateral flow test from Strategic Diagnostic Inc. (Newark, DE) prior from initiating the experiments. For both tests (choice and no choice) fresh leaf tissue was collected each day to assure freshness of the tissue. Leaf discs were cut using a number 13-cork borer that generates 1.7 cm diameter leaf discs.

Choice and No-Choice Tests

Choice tests were performed to test whether resistant and susceptible phenotypes discriminated between *Bt* and non-*Bt* plants and to determine if behavior was affected by exposure to *Bt* plants. For all experiments, BioServ clear 32 cell rearing trays (Frenchtown, NJ) were used; each half tray represented one phenotype. Individual wells

were 5.1 cm long, 3.8 cm width and 2.9 cm tall. Three replications were used for a total of 48 larvae per phenotype for the choice test and 24 larvae for the no-choice test. Replications were represented by randomly selecting neonates eclosing from a cohort of eggs laid on three different days to control for differences among sample cohorts (Robertson *et al*. 1995).

Fifteen ml per well of a solution with 10 g/ml of agar, 0.3 g/ml of sorbic acid and 1.7 g/ml of methyl paraben was dispensed to prevent leaf tissue degradation and contamination. Using a small spatula, the agar was scored and the leaf discs positioned vertically in the agar. Both tissues (TC1507 and isoline) were placed in each well for the choice experiment, and a single tissue type was placed in each well for the non-choice experiment. Leaf discs were placed facing each other in the choice experiment; the distance between leaf discs was approximately 1.5 cm. Leaf disc position per well was previously randomized using PROC RANK (SAS Institute 2011). One larva per well was transferred with a fine paintbrush, placed between discs and the wells were covered with Breathe Easy ® gas permeable sealing membrane for microtiter plates (USA Scientific, Orlando FL). Experiments were held at room temperature at 22°C ± 2°C and 30% ± 20% RH under a LD 14:10 h cycle.

The position of the larvae and mortality was recorded for five days. Behavior was categorized in the following way for the choice experiment: (1) on Cry1F corn (TC1507), (2) on isoline, (3) off tissue, (4) dead, and (5) missing; and for the no-choice experiment: (1) on plant tissue, (2) off tissue, (3) dead, and (4) missing. Data was collected every 30 minutes for the first seven hours, and after the first day data was collected three times a

day for five days (111 hours). After the sixth day, data were collected in the morning and surviving larvae were weighed.

Data Analysis

All analyses were performed using SAS software (SAS Institute 2011). The data for mortality and the location of the larvae were converted to proportions. Mortality was analyzed for the choice experiment to confirm the susceptibility of the phenotypes. Mortality was initially recorded at seven hours; so percent mortality was analyzed from seven to 111 hours. As a result of mortality occurring after seven hours, larval position was determined for the first seven hours. Missing larvae was less than 1% for all experiments. Dead and missing larvae were excluded from the larval position analysis. The proportion of the position of larvae was analyzed by generating two time points for each larva. The analysis was performed in this manner instead of generating proportions for all larvae per replication at each time point to avoid losing degrees of freedom. For each larva, the first time point consisted of the proportion of the position from 30 minutes to 3 hours and the second time point from 4 hours to seven hours. Proportions of larvae in the different positions were transformed by arcsine-square root (Martin and Bateson 2007, Prasifka *et al*. 2009) prior to analysis. The choice and no-choice experiments were analyzed independently using a repeated measures analysis of variance (RM-ANOVA) with the PROC GLIMMIX procedure and a Kenward-Rogers adjustment for degrees of freedom (version 9.3; SAS Institute 2011) (Prasifka *et al*. 2009). A test for equality of covariance (covtest) was used to test for homogeneity of variance. The main factors measured for the choice experiment were phenotype, time and location, and for the no-

choice experiment plant, phenotype and time. All interactions were taken into account for both analysis. Pairwise differences were assessed using least-square estimated means with the slice option and a Bonferroni adjustment for *P*-values using PROC GLIMMIX (SAS Institute 2011) (Prasifka *et al*. 2009).

Results

Choice Experiment

In choice experiments, mortality of *S. frugiperda* neonates exposed to Cry1F leaf tissue was observed within the first 24 hours and within 18 hours for *O. nubilalis* (Figure 1). Susceptibility of resistant and susceptible phenotypes was confirmed based on the observed differences in mortality during the exposure period. By the end of 111 hours 43% of susceptible FAW and 56% of susceptible ECB survived in the choice experiments. Surviving susceptible larvae were either severely stunted or had initiated feeding on isoline plants and were unaffected. Survival of the resistant phenotype for both species exceeded 90%.

The repeated measures analysis of the proportion of larvae at the various positions within the bioassay arena for neonate *S. frugiperda* (FAW) and *O. nubilalis* (ECB) phenotypes is presented in Table 1. The FAW analysis showed no significant differences between phenotypes or times but significant differences among position ($F = 87.91$; df = 1; $P < 0.001$). The proportion of larvae on TC1507 was significantly higher than the proportion of larvae on isoline ($t = 3.35$; df = 552; $P = 0.0026$) for both resistant and susceptible phenotypes. In addition, the proportion of larvae not observed on either leaf

disc was significantly lower than the proportion observed on TC1507 (t = 12.79; df = 552; P < 0.0001) and isoline (t = 9.43; df = 552; P <0.001). No significant interactions between factors were found in the repeated measures analysis for FAW.

The ECB repeated measures analysis showed a similar trend as there were no significant differences between phenotypes (F = 0.01; df = 1; P = 0.931) or times (F = 0.07; df = 1; P = 0.785), but there were significant differences among positions (F = 31.3; df = 1; P < 0.0001). In contrast to FAW, the proportion of larvae on TC1507 was significantly lower than on isoline (t =-0.2045; df = 516; P < 0.0001). In addition the proportion of ECB larvae on TC1507 (t = -7.85; df = 516; P < 0.0001) and isoline (t = -3.08; df = 516; P =0.007) was significantly lower than the proportion of larvae that were not observed on either leaf disc. The only significant interaction was time by location (F = 29.27; df = 1; P < 0.0001) and phenotype by location approached significance (F = 2.84; df = 2; P = 0.06).

The movement of susceptible and resistant phenotypes of first instars FAW and ECB during the first seven hours is presented in Figure 2. For both species, no clear differences were detected between phenotypes. Both resistant and susceptible FAW phenotypes exhibited less movement and rapid choice of host, while both ECB phenotypes spent more time moving between plant tissues.

No-Choice Test

The repeated measured analysis on the proportion of neonate *S. frugiperda* (FAW) and *O. nubilalis* (ECB) phenotypes on plant is represented in Table 2. Analysis of the FAW results indicated no significant differences at any factor and/or interaction. ECB

showed no significant differences by plant and time, but a significantly higher proportion of resistant larvae were observed on leaf discs of both TC1507 and isoline compared to susceptible larvae ($t = 2.12$; df = 180; $P = 0.036$). No significant interactions between factors were found in the repeated measures analysis for ECB.

The movement of the susceptible and resistant first instar FAW and ECB during the first seven hours of the no choice experiment is represented in Figure 3. FAW and ECB susceptible and resistant neonates exhibited similar behavior to that observed in the choice experiment. FAW exhibited less movement and more rapid choice, while ECB took longer to find the plant tissue and spent more time wandering in the bioassay arena. Additionally, susceptible larvae of both species tended to abandon TC1507 corn tissue, while resistant larvae seemed to not be affected by the presence of Cry1F. However, significant differences between phenotypes were only observed for ECB and not in FAW (Table 2).

Discussion

Results from both choice and no-choice experiments indicate differences in the behavior of neonate *S. frugiperda* and *O. nubilalis*. Most *S. frugiperda* larvae tend to select a plant within the first 30 minutes and remain on the chosen tissue regardless of Cry1F presence. In contrast, *O. nubilalis* displayed inconsistent movement on and off plant tissue in both experiments, and significant differences between phenotypes were observed in the no choice experiment. However, increased movement in *O. nubilalis* might be an artifact of the higher number of generations the strains have been reared in the laboratory resulting in less recognition of corn as a suitable host (Visser 1986, Stuhl

et al. 2008). Additional differences were found regarding the preference of corn tissue in the choice experiment. *S. frugiperda* preferred Cry1F corn tissue while *O. nubilalis* more frequently selected isoline corn. No differences in preferences between diets with or without *Bt* toxins have been previously described in other Lepidoptera species (Stapel *et al*. 1998, Prasifka *et al*. 2009). Preference results could also have been an artifact of slight differences in plant quality (Goverde and Erhardt 2003) since the ECB and FAW experiments could not be conducted at the same time with identical plant material; and/or to other innate behavioral factors not associated with the nutritional quality of the host (Thompson 1988, Berdegué *et al*. 1998).

Although differences between species were easy to detect, comparisons between susceptible and resistant phenotypes within each species were more difficult to assess. No significant differences among phenotypes were observed in the choice experiment for either species. Similarly, in the *S. frugiperda* no choice experiment, no differences between phenotypes were observed. For *O. nubilalis* no-choice experiment, significant differences were observed, with resistant larvae spending more time on both types of plant tissue than susceptible individuals. Although significant differences were not observed in the majority of the experiments, a small percentage of susceptible larvae of both species abandoned corn tissue expressing Cry1F. However, exposure to Cry1F plant tissue did not lead to avoidance (Lockwood *et al.* 1984, Stapel *et al*. 1998). In contrast, resistant larvae did not exhibit improved ability to reduce or avoid exposure, but seemed unaffected by the presence of Cry1F (Stapel *et al.* 1998, Prasifka *et al*. 2009). The lack of a behavioral response of resistant larvae to Cry1F corn might be explained by the ability

of the larvae to overcome the toxin and by the absence of a fitness cost linked to Cry1F resistance in both species (Pereira *et al*. 2009, Chapter 3).

The tendency of susceptible *O. nubilalis* larvae to stay off leaf material might be an indication of irritability generated by Cry1F ingestion as described for other Lepidoptera species exposed to Cry toxins (Berdegué *et al*. 1996, Stapel *et al*. 1998, Prasifka *et al*. 2009). Consequently, it is possible that susceptible first instar *O. nubilalis* could move from a Cry1F expressing plant to a non-*Bt* plant and recover from intoxication (Stapel *et al*. 1998, Li *et al*. 2006). Studies with *S. exigua* feeding on *Bt*-treated diets and *T. ni* feeding on *Bt* cotton indicate that larvae that fed on a mixture of non-*Bt* and *Bt* were able to survive (Stapel *et al*. 1998, Li *et al*. 2006), increasing the likelihood of heterozygote survival and potentially accelerating the evolution of resistance (Mallet and Porter 1992, Davis and Onstad 2000). Increased movement of *O. nubilalis* in response to Cry1F exposure indicates that current refuge configurations (i.e. blocks or strips) are more suited for this insect (Ross and Ostlie 1990).

In contrast to reports of behavior of other Lepidoptera species, the majority of susceptible *S. frugiperda* larvae remained on selected plant tissue regardless of toxin expression. Previous larval preference studies of corn and stargrass, *Cynodon nlemfuensis* Vanderyst, by *S. frugiperda* corn and rice strains indicated that neonates of both strains feed on the plant type that was encountered first and a substantial number of larvae remained on the selected plant tissue (Stuhl *et al.* 2008). These results suggest that the innate behavior of *S. frugiperda* neonates is to remain on the first plant tissue found. For *S. frugiperda,* more rapid host selection and reduced movement of early instars away from *Bt* corn may have important implications for refuge design. If these traits are similar

under field conditions, the use of seed mixtures or refuge in a bag might provide a suitable strategy for *S. frugiperda* because increased movement due to exposure to Cry1F might not be of concern.

Prasifka *et al*. (2009) reported irritability of *O. nubilalis* neonates to Cry1Ab over 72 hours of exposure with different percentages of Cry1Ab corn tissue incorporated into artificial diet. The experiments performed in this study used corn tissue expressing a high dose of Cry1F that generated significant mortality within the first 24 hours of exposure. For that reason, observations over a prolonged period of time were not possible. Additional experiments exposing larvae to sublethal Cry1F concentrations could generate a better interpretation of the effect of Cry1F on movement of susceptible and resistant larvae. Finally, similar experiments with later instar larvae will be critical to provide a more complete interpretation of the effects on movement to Cry1F feeding.

This study represents the first step toward understanding the effects of Cry1F in *S. frugiperda* and *O. nubilalis* larval behavior. However, further greenhouse and field experiments are necessary to provide a more complete understanding of the effect of Cry1F on movement of susceptible and resistant larvae, and the differences between *S. frugiperda* and *O. nubilalis*. Despite the knowledge that laboratory behavior experiments are difficult to extrapolate to field behaviors (Prasifka *et al*. 2009); the apparent differences in the behavior of *O. nubilalis* and *S. frugiperda* exposed to Cry1F corn suggest that not all Lepidoptera species perform equally, and generalizations in behavior might not always be accurate. Understanding behavioral differences between species could help us to develop better and more flexible resistance management strategies (EPA 1998, Onstad *et al*. 2011).

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Tables

Table 1. *F*-test values and probabilities for the three-factor repeated measures analysis on the proportion of *S. frugiperda* (FAW) and *O. nubilalis* (ECB) resistant and susceptible phenotypes at each location in a choice experiment.

Insect	Source	df	F	P
FAW	Phenotype	1	0	0.924
	Time	1	0	0.958
	Location	1	27.11	<0.0001*
	Phenotype x Time	2	0	0.981
	Phenotype x Location	2	1.63	0.182
	Time x Location	2	0.30	0.881
	Phenotype x Time x Location	2	0.62	0.114
ECB	Phenotype	1	0.01	0.931
	Time	1	0.07	0.785
	Location	1	31.30	<0.0001*
	Phenotype x Time	2	0.02	0.887
	Phenotype x Location	2	2.84	0.06
	Time x Location	2	29.27	<0.0001*
	Phenotype x Time x Location	2	0.45	0.636

Measurements were obtained from observations of 42 to 48 individuals per phenotype.

* Significantly different ($P > 0.05$).

Table 2. *F*-test values and probabilities for the three-factor repeated measures analysis on the proportion of resistant and susceptible neonate *S. frugiperda* (FAW) and *O. nubilalis* (ECB) phenotypes on plant in the no-choice experiment.

Insect	Factor	df	F	P
FAW	Plant	1	1.74	0.189
	Phenotype	1	0.02	0.89
	Time	1	2.11	0.148
	Phenotype x Plant	1	2.19	0.14
	Plant x Time	1	0.89	0.348
	Phenotype x Time	1	0.05	0.831
	Phenotype x Plant x Time	1	0.13	0.716
ECB	Plant	1	0.08	0.776
	Phenotype	1	4.47	0.036*
	Time	1	1.91	0.169
	Phenotype x Plant	1	0.18	0.67
	Plant x Time	1	1.41	0.237
	Phenotype x Time	1	0.31	0.576
	Phenotype x Plant x Time	1	0	0.966

Measurements were obtained from observations of 22 to 24 individuals per phenotype by type of plant tissue (TC1507 and isoline).

* Significantly different ($P > 0.05$).

Figures

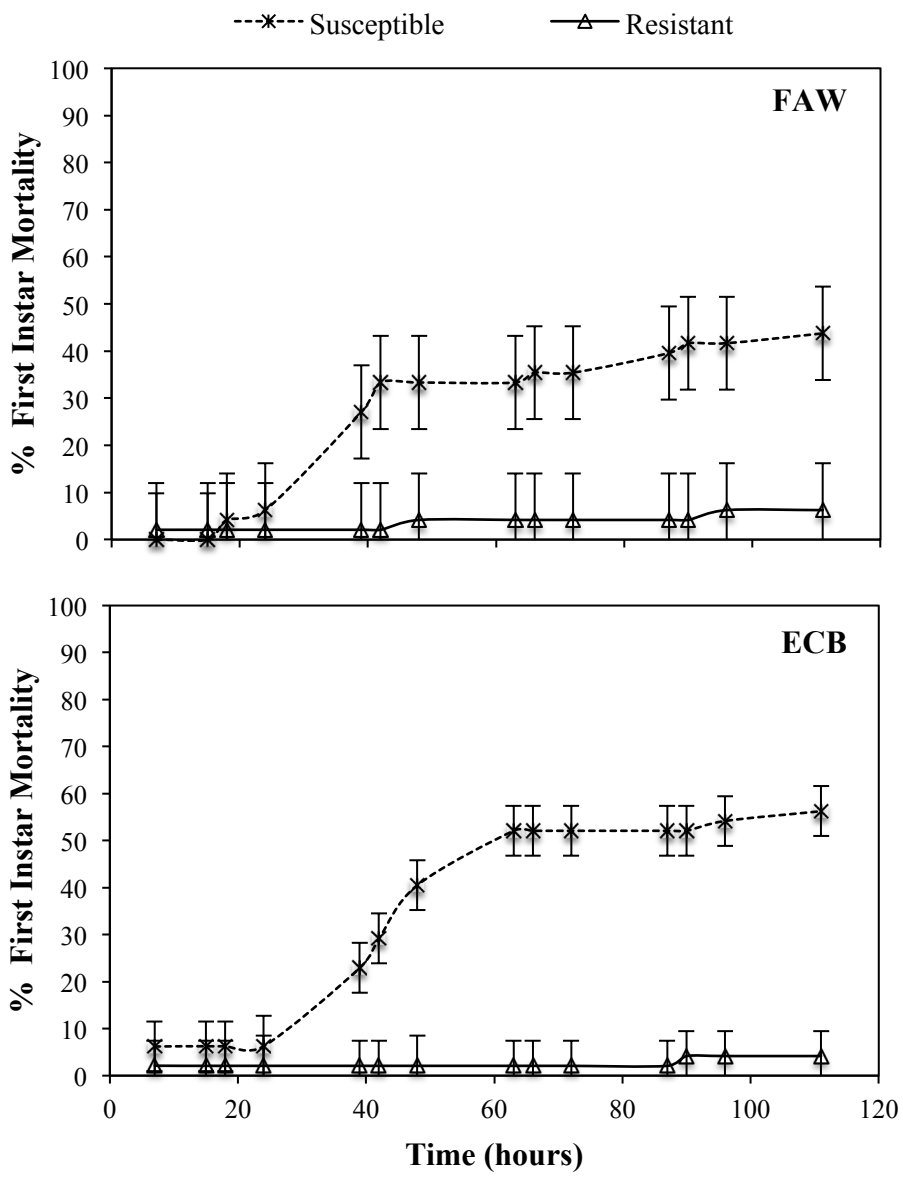

Figure 1. Mortality of neonate *S. frugiperda* (FAW) and *O. nubilalis* (ECB) phenotypes found in the choice experiment (n = 48). Error bars represent standard errors.

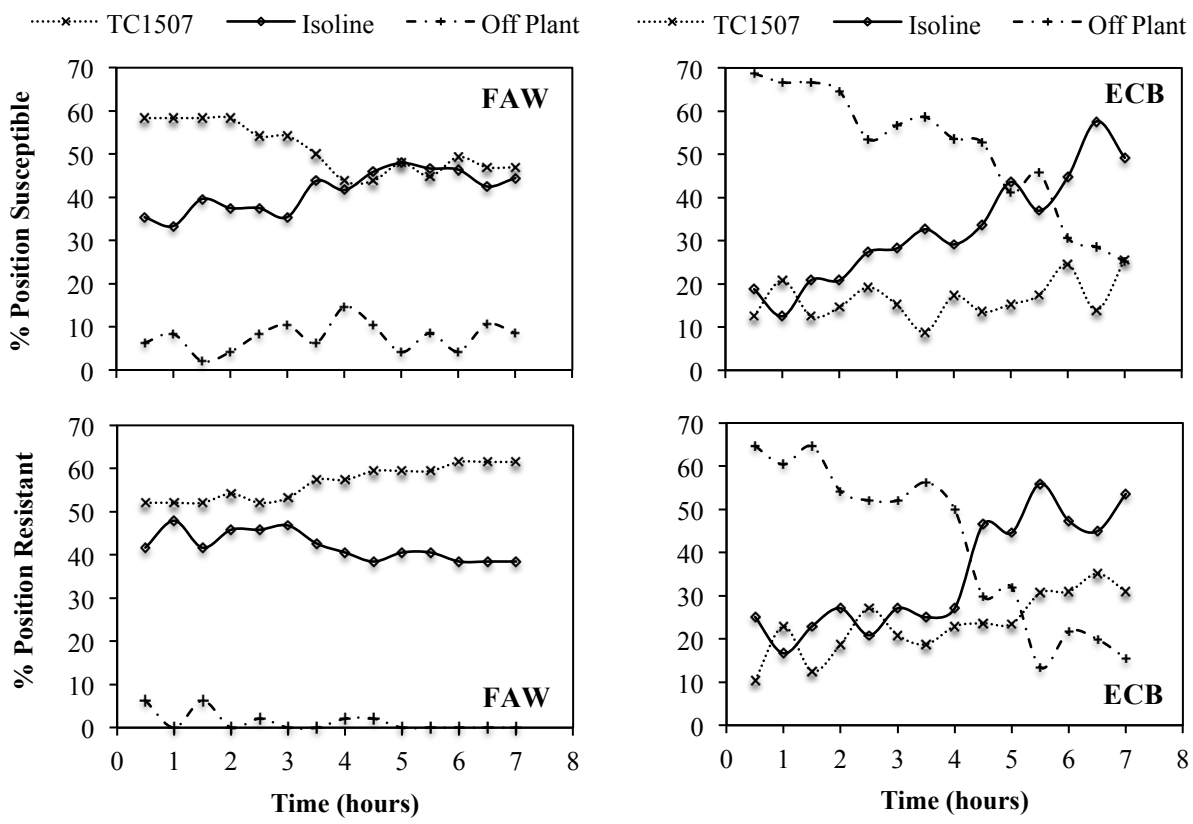

Figure 2. Choice experiment percentage of susceptible and resistant neonate *S. frugiperda* (FAW) and *O. nubilalis* found on TC1507 corn tissue, on isoline and off plant (n = 42 - 48).

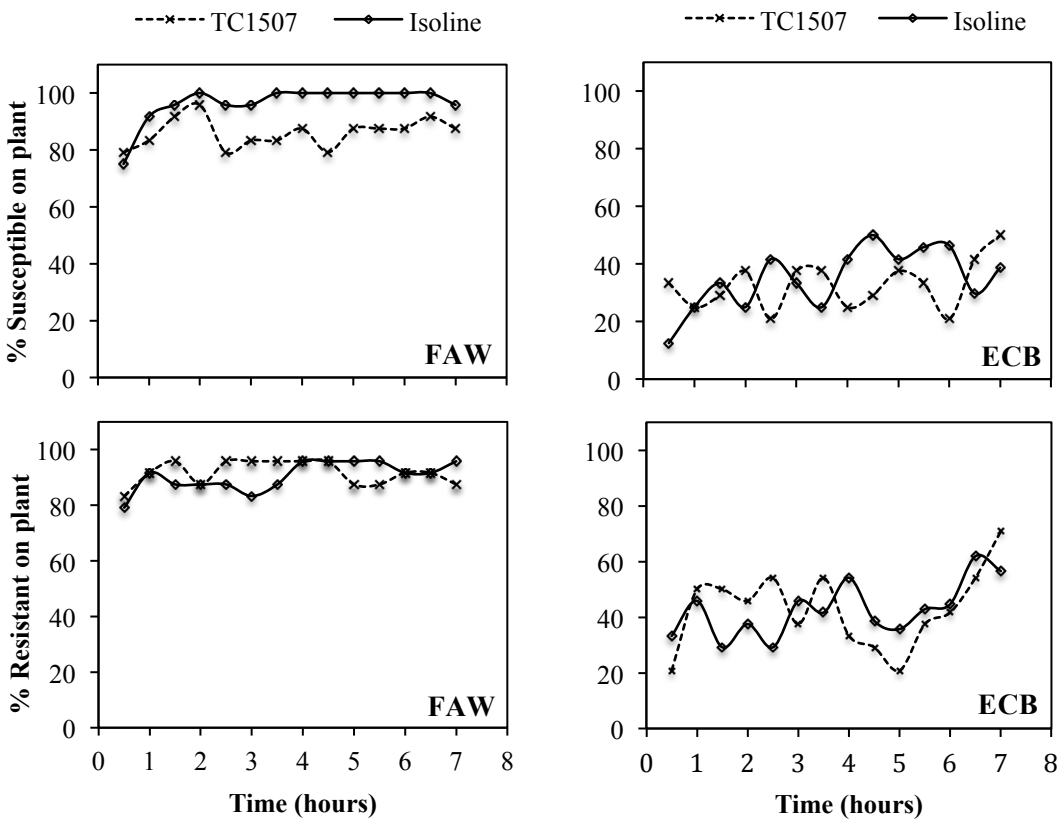

Figure 3. No choice experiment percentage of susceptible and resistant neonate *S. frugiperda* (FAW) and *O. nubilalis* (ECB) on TC1507 corn and isoline (n = 22 - 24).

CHAPTER 5: Summary and Conclusions

Transgenic corn, *Zea maize* L., expressing Cry1F protein from *Bacillus thuringiensis* Berliner (*Bt*) has been registered for *Spodoptera frugiperda* (J. E. Smith) control since 2003. Corn hybrids containing Cry1F are marketed as Herculex® I *Insect Protection* (transformation event TC1507). Even though TC1507 corn has been commercially available in the United States since 2003, this even has been grown in Puerto Rico since 1998 for experimental plots, hybrid development and parental seed production (Buntin 2008). Unexpected damage to Cry1F corn was reported in 2006 in Puerto Rico and Cry1F resistance in *S. frugiperda* was documented (Matten *et a*l. 2008, Tabashnik *et al*. 2009). Storer *et al.* (2010) confirmed the high-level of resistance to Cry1F, and described resistance as autosomal and recessive. Field resistance occurred after four years of commercialization, making it the fastest documented case of field-evolved resistance to a *Bt* crop and the first documented case of field failure associated with insect resistance to a *Bt* crop leading to withdrawal of a *Bt* crop from the marketplace (Tabashnik *et al*. 2009, EPA 2010).

S. frugiperda field resistance gives us an opportunity to more rigorously test the correspondence between evidence and theory, and an opportunity to validate and improve current insect resistant management strategies. Therefore, additional studies considering different aspects of Cry1F resistant alleles (e.g. fitness, behavior, migration) in *S. frugiperda* will allow us to have a better understanding of how resistance evolved in Puerto Rico and to determine the risk of field resistance in the United States.

The research described in this dissertation addressed several aspects regarding *S. frugiperda* resistance to Cry1F corn in Puerto Rico: (1) Quantification of resistance levels using bioassays with Cry1F in artificial diet and determination of the genetic basis of resistance (i.e., dominance, sex-linkage, and number of resistance genes); (2) Identification of cross-resistance to other *Bt* toxins with lepidopteran activity; (3) Determination of the frequency of Cry1F resistance alleles in United States populations where resistance has not been previously reported (Florida and Texas); (4) Identification of the potential effects of resistance on reproductive and physiological fitness; and (5) Examination of the effects of Cry1F resistance on *S. frugiperda* and *O. nubilalis* neonate movement.

The quantification of resistance level, inheritance of resistance, cross-resistance and frequency of resistant alleles in Florida and Texas was described in Chapter 2. Cry1F bioassays indicated that the *S. frugiperda* Cry1F resistant strain from Puerto Rico displayed >387-fold resistance. Concentration-response bioassays of reciprocal crosses of resistant and susceptible parental populations indicated that resistance is recessive and autosomal. Bioassays of the backcross of the F_1 generation crossed with the resistant parental strain suggest that a single locus is responsible for resistance. In addition, cross-resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry2Aa and Vip3Aa was assessed in the Cry1F resistant strain. There was no significant cross-resistance to Cry1Aa, Cry1Ba and Cry2Aa, although only limited effects were observed in the susceptible strain. Vip3Aa was highly effective against susceptible and resistant insects indicating no cross-resistance with Cry1F. In contrast, significant cross-resistance (< 20-fold) was observed for both Cry1Ab and Cry1Ac. Because the resistance was recessive and conferred by a

single locus, an F_1 screen was used to measure the frequency of Cry1F resistant alleles from populations of Florida and Texas in 2010 and 2011. A frequency of 0.13 was found in Florida, while Texas populations had a resistant allele frequency of 0.015. Results indicated that resistant alleles could be found in continental United States populations although there have been no reports of reduced efficacy of Cry1F-expressing plants against *S. frugiperda*. Puerto Rico populations tested in 2010, 2011 and 2012 with the diagnostic concentration showed that the frequency of Cry1F resistance in Puerto Rico remains high although a low frequency of susceptible alleles may exist.

The fitness studies (Chapter 3) provided comparisons of life-history traits and population growth rates of homozygous susceptible, heterozygous and homozygous resistant *S. frugiperda*. Results were used to determine if *S. frugiperda* Cry1F resistance is associated with fitness costs. Major fitness costs were not apparent in either heterozygotes or homozygous resistant insects. However, there was a slight indication of hybrid vigor in the heterozygotes. Additionally, results from a population cage experiment performed with two lines followed for seven generations indicated that the frequency of resistant alleles decreased slightly. In spite of this decline in resistance alleles, it is difficult to determine if it was related to an undetected fitness cost or the result of random drift. The lack of strong fitness costs may affect initial frequency of resistant alleles in field populations and persistence in resistant populations (e.g. Puerto Rico).

Results from the behavior study performed with Cry1F resistant *O. nubilalis* and *S. frugiperda* (Chapter 4) suggested no strong differences between resistant and susceptible phenotypes in both species. However, the *O. nubilalis* no-choice experiment

indicated that susceptible neonates spent less time on plant than resistant neonates, suggesting that irritability generated by Cry1F ingestion may occur (Berdegué *et al*. 1996, Stapel *et al*. 1998, Prasifka *et al*. 2009). In addition, behavioral differences were observed between species; *O. nubilalis* exhibited increased mobility, while *S. frugiperda* tended to stay on the selected leaf disc regardless of the presence of *Bt*. *S. frugiperda* behavior suggests that the use of refuge in a bag might be a suitable strategy for this insect. This study represents the first step toward understanding the effects of Cry1F on *S. frugiperda* and *O. nubilalis* neonate behavior. Despite the recognition that laboratory behavior experiments are difficult to extrapolate to field behaviors (Prasifka *et al*. 2009), the apparent differences in the behavior of *O. nubilalis* and *S. frugiperda* exposed to Cry1F corn suggest that not all Lepidoptera species perform equally. Indicating that generalizations in behavior might not always be accurate and more larval movement studied of pests targeted with *Bt* crops might be necessary. Future understanding of behavioral differences between species could help us to develop better and more flexible resistance management strategies (EPA 1998, Onstad *et al*. 2011).

The studies described in this dissertation provide information on different aspects of Cry1F resistance in *S. frugiperda* that have not been previously described. The occurrence of resistant alleles in Florida and Texas and the lack of fitness cost in resistant homozygotes indicate that there is a risk of resistance evolution in the continental United States and persistence in resistant populations (e.g. Puerto Rico) and that resistance alleles were present prior to selection. Lack of fitness cost might affect the durability of Cry1F corn and make remediation and management tactics more challenging. In consequence implementation of monitoring programs together with the investigation of

reports of unexpected damage to Cry1F-expressing maize are suggested as a priority. If reduction of product performance is linked to changes in allele frequency in the continental United States, actions should be taken to limit survival of resistant insects and slow or prevent their spread (Siegfried *et al.* 2007). Additionally, cross-resistance results from this study suggest that the use of a pyramided product expressing Vip3Aa could help to control and prevent the spread of *S. frugiperda* Cry1F-resistant alleles.

Information of species that have evolve resistance in the field like *S. frugiperda* can contribute to the development of better risk assessments, improve predictions of resistance to *Bt* crops in other Lepidoptera and maximize the benefits of current and future generations of transgenic crops. Specifically, information derived from this dissertation could guide resistant management strategies for Latin America were *S. frugiperda* is an important pest of corn and cotton. Planned deployment of *Bt* crops in Latin America suggest the need for resistant management programs designed for tropical areas where crop production is year round and pest pressure is continuous.

APPENDIX A: *S. frugiperda* rearing protocol

FAW REARING PROTOCOL

Egg Production

- Harvest eggs daily to avoid egg cannibalism by newly emerged neonates.
- Transfer eggs to petri dishes with filter paper moistened with ~1 ml mold inhibitor (recipe to follow) and seal with parafilm. During peak egg-laying times (day 4 to 9 after the cage starts laying eggs), divide eggs masses into two petri dishes per colony.
- Label petri dishes with colony designation, generation (remember this is F+1 from the generation in the cage), date that the cage started laying eggs and date that the eggs were collected.
- Clean lab bench with 70% ethanol between colonies to avoid cross-colony contamination.
- Place petri dishes in growth chamber at 14°C, 24 hours light.
- Eggs should be used within 10 days after egg collection date; after this time, egg viability decreases.
- Eggs should be taken out of the chamber to hatch 2-3 days before day of use during the summer and 3-4 days during the winter. Eggs will turn dark gray when they are close to hatching. If eggs have not hatched by the 5^{th} day, they are likely not viable.

- When petri dish with eggs is removed from chamber, label the dish with date of removal from chamber.
- One 9-cm diameter petri dish with 1000-1500 eggs is generally enough to perpetuate a colony.

Larval rearing

- Cook 250 ml of larval diet (recipe to follow), pour into a ~20 cm diameter pan and allow to dry for at least one hour. Once the diet is completely dry, shred diet into the pan.
- Label pan with colony designation, generation and date of transfer.
- Transfer hatched neonates to shredded diet and seal lid on pan with tape to avoid larvae escaping.
- Place pan in larval rearing room. Rearing room is kept at 28-30°C and 24 hours light.
- After 5 to 7 days, larvae will be at third to fourth instar and need to be transferred to avoid cannibalism.
- Transfer individual larvae from pan to 1 oz. translucent, polystyrene soufflé portion cups (Solo Cup Company, Lake Forest, IL). Cook enough larval diet to fill each cup with 4.5 ml of diet dispensed with an Eppendorf Multipette® plus (Eppendorf, New York). Allow diet to dry, and score with a clean spatula to permit easier larval feeding.
- For each colony, a minimum of 200 larvae, and preferably 300, should be transferred to cups to maintain genetic variability and avoid colony collapse.

- Transfer larvae gently to cups with clean forceps. Select large larvae first to maintain vigor in the colonies.
- Seal cups with matching lids (Solo Cup Company, Lake Forest, IL). Pierce all lids 3 times with thumbtack to allow for ventilation. Lids can be cleaned with a 20% bleach solution and reused. If larvae show symptoms of disease, lids should be discarded.
- Place sealed cups in cup holder trays for easier manipulation and label trays with colony designation, generation and date of transfer.
- Place trays in larval rearing room.

Pupation

- Check for pupation one week after larvae have been transferred to cups.
- When majority of larvae have pupated (7-12 days after transfer), place pupae into one "honeymoon cage" (description below) with one petri dish removed, or an open plastic container. At the bottom of the honeymoon cage or plastic container, place a layer of slightly moistened wood shavings, then add pupae and cover pupae with another layer of moistened wood shavings. Limit amount of water added to wood shavings, as excess moisture will generate fungi.
- "Honeymoon cages" are made of 27-gauge hardware cloth secured in the shape of cylinder (4.2 cm tall) by staples; ends of the cylinder are covered by 33-mm diameter disposable petri dishes (Appendix B).
- Place honeymoon cage or plastic container with pupae in large, wired hermit crab cage (31 x 23 cm) (Florida Marine Research, Sarasota FL). Outside of wired

section of the cage should be wrapped with wax paper to allow for egg laying (Appendix B). Label cage with colony designation, generation, and leave space on label to write the first date of egg production.

- Place cage in moth room kept at 30°C during the day and 24°C during the night with a 14:10 (L:D) photoperiod.
- Check for adult emergence daily. When first moth emerges, place a cotton pad in a 9 cm diameter petri dish (Fisherbrand, Waltham, MA), soak pad with adult diet (see recipe below) and place in the cage. Cotton pad should be cleaned with water daily and replenished with diet.
- Allow adult moths to mate and lay eggs on wax paper.
- Harvest eggs daily for at least 10 days or until females are done laying eggs.
- To kill colony, place it in a freezer at -4 C for at least one hour to exterminate all moths.
- Clean all rearing components and place them in a 20% bleach solution for one day to sanitize. Rinse with water and allow drying.

Egg Mold Inhibitor

- Water 100 ml
- Propionic acid 0.15 ml
- Phosphoric acid 0.02 ml

Larval Diet (1L)

The diet with best performance was general Lepidoptera diet from BioServ (Frenchtown, NJ). Note: High moisture content in the diet may cause disease and colony collapse.

- Water 875 ml
- Dry mix 144 g
- Agar 19 g

Add agar to the water and mix, then cook in microwave or kettle. If cooked in microwave, allow the mixture to boil, then remove from the microwave and mix thoroughly, boil again and add dry mix. Wait 5 minutes, mix again and let mixture stand at room temperature until dry. If cooked in kettle, allow the mixture to boil, then transfer to another container to be dispensed or to allow it to dry.

Adult Diet (355 ml)

- Light beer (example Milwaukee's best) 355 ml (1 can)
- Ascorbic acid 0.53 g
- Aureomycin 0.18 g
- Propionic acid 750 µl
- Vitamin mix 0.9 g

APPENDIX B: *S. frugiperda* rearing pictures

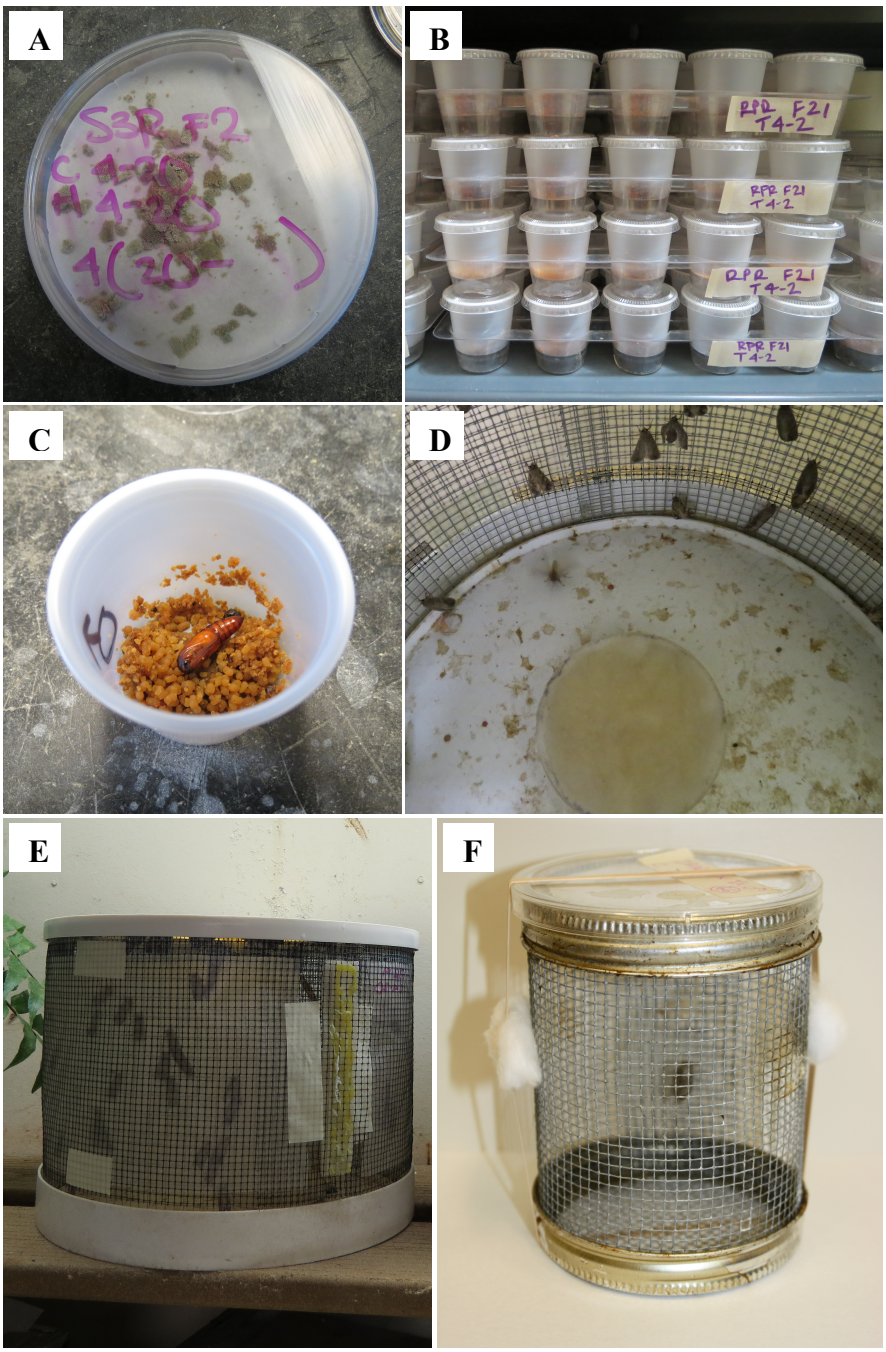

Figure B. *S. frugiperda* rearing, (A) petri dish with harvested eggs, (B) cups with individual larvae, (C) pupae in individual cup, (D) pad with adult diet in cage, (E) mass rearing cage (hermit crab cage), and (F) "honeymoon cage".

APPENDIX C: *S. frugiperda* pupae and adults sex identification

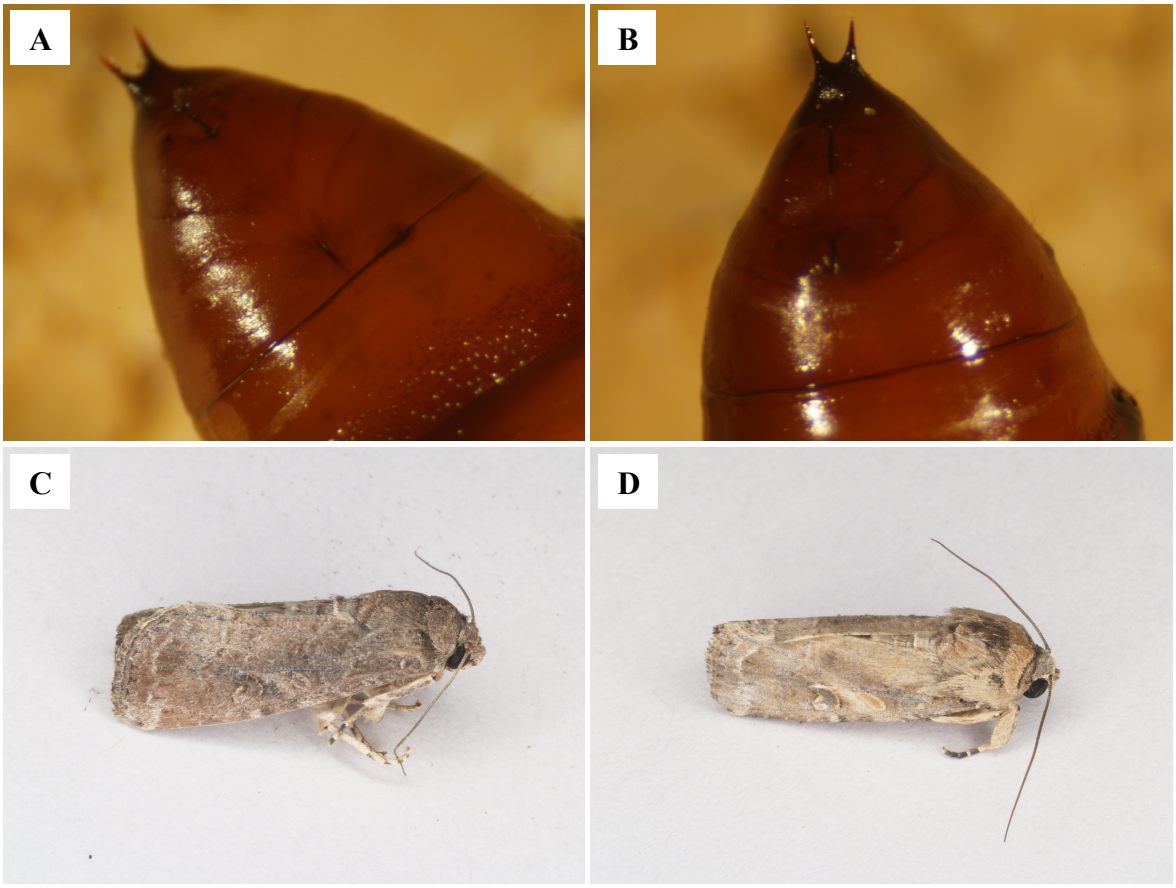

Figure C. *S. frugiperda* pupae and adults sex identification. (A) Female pupae, genital opening located in segment eight; (B) male pupae, genital opening located in segment nine; (C) female adult, forewings are less distinctly marked, ranging from uniform grayish brown to a fine mottling of gray and brow; and (D) male adult, forewings generally shaded gray and brown, with triangular white spots at the tip and near the center of the wing (Capinera 2000).